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Title: Studies to Select Appropriate Nonpathogenic Surrogate *Escherichia coli* Strains for Potential Use in Place of *Escherichia coli* O157:H7 and *Salmonella* in Pilot Plant Studies

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Studies To Select Appropriate Nonpathogenic Surrogate *Escherichia coli* Strains for Potential Use in Place of *Escherichia coli* O157:H7 and *Salmonella* in Pilot Plant Studies†

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ABSTRACT

The response of a potential nonpathogenic surrogate organism to a particular treatment should closely mimic the response of the target pathogenic organism. In this study, growth characteristics (generation time, lag phase duration, and maximum population), pH at stationary phase, and survival characteristics (level of attachment and survival on apple surfaces, resistance to hydrogen peroxide decontamination treatments, and thermal resistance at 60°C) of 15 nonpathogenic generic *Escherichia coli* strains and one nonpathogenic *E. coli* O157:H43 strain were compared with those of two *E. coli* O157:H7 strains and two *Salmonella* strains. Few differences in growth characteristics or pH at stationary phase were evident between nonpathogenic and pathogenic strains tested. However, considerably more separation among strains was seen following investigation of survival characteristics. *E. coli* ECRC 97.0152, which does not contain genes encoding for known virulence factors associated with *E. coli* O157:H7, appears to be a good surrogate candidate, with growth and survival characteristics similar to those of *E. coli* O157:H7 strains. The less heat-resistant surrogate strains *E. coli* NRRL B-766 and NRRL B-3054 and *E. coli* ATCC 11775, ATCC 25253, and ATCC 25922 may be used when attempting to model the heat resistance of *Salmonella* Montevideo G4639 and *Salmonella* Poona RM 2350, respectively. These surrogate strains may be useful for evaluating the efficacy of intervention steps in reducing populations of selected strains of *E. coli* O157:H7 and *Salmonella* in processing environments where these pathogens cannot be introduced.

Researchers in the field of applied microbial food safety have many factors to consider when designing validation and challenge studies. Often, the goal of such research is to characterize the behavior of a particular pathogen in a target food of food processing step with a view to developing a way of eliminating or controlling pathogen proliferation. Although the use of the intended target pathogen would be ideal, researcher safety concerns must also be considered. In many instances, researchers find it necessary to use a nonpathogenic surrogate organism in place of the target pathogenic organism. Such studies are inherently limited because surrogate organisms may not behave in a manner identical to that of their pathogenic counterparts. Nonpathogenic surrogate organisms do not have the virulence genes of wild-type outbreak strains, i.e., the type of strains whose behavior and responses the research is attempting to reproduce. Pathogenic strains also may have unique characteristics that contribute to their virulence. For example, *Escherichia coli* O157:H7 is highly tolerant of acidic conditions *(8)*, a factor that has been cited as contributing to the virulence of this pathogen by permitting survival in the low pH of the stomach *(12)*. The U.S. Food and Drug Administration *(11)* defined a surrogate microbe as "a nonpathogenic species and strain responding to a particular treatment in a manner equivalent to a pathogenic species and strain. The surrogate allows biological verification of the treatment without introducing pathogens into a food processing area." Therefore, potential nonpathogenic surrogate organisms should be characterized prior to use in validation and challenge studies and should be negative for virulence factors of target pathogens relevant to the food system of interest.

Our laboratory is engaged in pilot plant studies to characterize the efficacy of various washing and sanitizing treatments in reducing populations of pathogenic microflora, specifically *E. coli* O157:H7 and *Salmonella*, on fresh fruit and vegetables. Because these organisms cannot be introduced to the pilot plant in its present configuration because of concerns for the safety of equipment operators or other personnel in the pilot plant area, nonpathogenic organisms are needed. The aim of the present study was to match the growth, survival, and thermal resistance characteristics of 15 nonpathogenic generic *E. coli* strains and one nonpathogenic O157:H43 *E. coli* strain with characteristics of two strains each of *E. coli* O157:H7 and *Salmonella*. All four pathogenic strains were previously associated with foodborne outbreaks in produce or apple cider. Growth response and thermal inactivation of bacteria were previously shown

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† Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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TABLE 1. *Bacterial strains*

Strain[a]	Source
E. coli O157:H43[b]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 96.0509[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 97.0147[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 97.0152[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 97.0190[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 99.0512[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 99.1232[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ATCC 11775[d]	Dr. P. Fratamico, USDA-ARS-ERRC, Wyndmoor, Pa.
E. coli ATCC 25253	Dr. R. Merker, FDA-CFSAN, Washington, D.C.
E. coli ATCC 35695	Dr. R. Merker, FDA-CFSAN, Washington, D.C.
E. coli ATCC 25922	Dr. W. Fett, USDA-ARS-ERRC, Wyndmoor, Pa.
E. coli NRRL B-766	Dr. L. K. Nakamura, USDA, National Center for Agricultural Utilization Research (NCAUR), Peoria, Ill.
E. coli NRRL B-2783	Dr. L. K. Nakamura, USDA, NCAUR, Peoria, Ill.
E. coli NRRL B-3054	Dr. L. K. Nakamura, USDA, NCAUR, Peoria, Ill.
E. coli NRRL B-3704	Dr. L. K. Nakamura, USDA, NCAUR, Peoria, Ill.
E. coli NRRL B-14573	Dr. P. Fratamico, cider outbreak strain
E. coli O57:H7 SEA 13B88	Dr. M. Lytle, Oklahoma State Department of Health, Oklahoma City; cider outbreak strain
E. coli O157:H7 OK	Dr. W. Fert, cantaloupe outbreak strain
Salmonella Poona RM 2350	Dr. L. Beuchat, University of Georgia, Griffin; tomato outbreak strain
Salmonella Montevideo G4639	

[a] ECRC, *E. coli* Reference Center, University Park, Pa.; ATCC, American Type Culture Collection; NRRL, Northern Regional Research Laboratory, Peoria, Ill.
[b] Nonpathogenic strain.
[c] All O55 strains.
[d] Contains green fluorescent protein on pGFPuv plasmid.

to be dependent on environmental parameters such as the growth medium *(2, 3, 5)*. In this study, the growth profiles and thermal resistance of all strains were examined following incubation in five typical growth media. Ongoing studies at our laboratory concern novel methods of decontaminating fruit with H_2O_2 and other antimicrobial agents; therefore, the level of attachment of the test strains to apple surfaces and their subsequent reduction by washing with a H_2O_2 solution were also investigated. Our goal was to identify a nonpathogenic surrogate that would not pose a health hazard and whose behavior closely resembled that of the pathogens represented under the parameters examined.

MATERIALS AND METHODS

Strains. Fifteen non-O157:H7 *E. coli* strains, one nonpathogenic *E. coli* O157:H43 strain, two *E. coli* 0157:H7 outbreak strains, and two *Salmonella* outbreak strains were used (Table 1). *E. coli* O157:H43, a nonpathogenic mutant of O157:H7, was included for comparison purposes only and was not considered as a possible surrogate. All strains were stored in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) containing 20% (vol/vol) glycerol at −80°C. Working stocks were kept on tryptic soy agar (TSA; Difco, Becton Dickinson) slants containing 0.6% (wt/vol) yeast extract (Difco, Becton Dickinson) at 4°C for 2 weeks.

All *E. coli* strains were confirmed by streaking on eosin methylene blue agar (Difco, Becton Dickinson) on which *E. coli* produces a green sheen with reflected light. *E. coli* O157:H7 and O157:H43 colonies from each study were further identified using the RIM *E. coli* O157:H7 latex agglutination test (Remel, Lenexa, Kans.), which identifies the O157 and H7 antigens. *Salmonella* strains were confirmed using a commercial latex agglutination test (Oxoid, Basingstoke, UK), which identifies the flagellar antigens.

All potential surrogate strains included in the study were submitted to the Gastroenteric Disease Center (Wiley Lab, Pennsylvania State University, University Park, Pa.) and tested for the presence of genes encoding for known virulence factors associated with *E. coli* O157:H7: (i) STa, heat stable toxin; (ii) STb, heat stable toxin; (iii) LT, heat labile toxin; (iv) SLT-I, Shiga-like toxin I; (iv) SLT-II, Shiga-like toxin II; (v) K99, fimbrial adhesion factor; (vi) *eae*, attaching and effacing gene; (vii) F1845, fimbrial gene; and (viii) CS31A, attachment factor.

Preparation of growth media. Five different growth media were prepared according to the manufacturers' instructions: (i) TSB (standard formulation contains 0.25% glucose); (ii) TSB+G (TSB supplemented with glucose [Sigma; St. Louis, Mo.] to a final concentration of 1% [wt/vol]); (iii) TSB−G (TSB with no glucose; Difco, Becton Dickinson); (iv) TSB−GHCl (TSB−G adjusted to pH 5.0 with 0.1 M HCl); and (v) brain heart infusion broth (BHI; Difco, Becton Dickinson). Initial pH of uninoculated TSB, TSB−G, TSB+G, and BHI was 7.4 to 7.6. BHI and TSB were chosen as typical growth media. TSB+G was included because it has been reported that the acid tolerance of *E. coli* O157:H7 is enhanced during growth in the presence of glucose in this manner because of adaptation to gradual production of acid by the metabolizing cells *(6)*. However, such growth conditions can result in a significant amount of injury to growing cells *(6, 14)*, which may impair their subsequent responses to stress. Therefore, the magnitude of the effect of glucose and acidity was investigated by growing the cells in TSB that contained no glucose (TSB−G) and by growing the cells in TSB−G in which HCl (an inorganic acid) was present as the acidulent (TSB−GHCl). The pH of each

broth was measured in triplicate after preparation and after cell growth of each strain reached the stationary phase.

Growth characteristics. Individual strains were grown in 10 ml of TSB at 37°C for 16 h with shaking at 75 rpm. The active culture was then diluted 1:100 with sterile 0.1% (wt/vol) peptone water (PW; Difco, Becton Dickinson), and 1 μl of this dilution was transferred to triplicate 30-ml volumes of each of the five growth media (TSB, TSB+G, TSB−G, TSB−GHCl, and BHI) in 250-ml Erlenmeyer flasks. The flasks were incubated at 37°C with shaking at 75 rpm. Populations were enumerated by aseptically removing a 0.1-ml aliquot of the culture at 0, 1, 2, 4, 6, 8, 10, and 24 h, diluting in 0.1% (wt/vol) PW as necessary, and surface plating on TSA. TSA plates were incubated overnight at 37°C prior to counting. Growth curves were generated by fitting the Gompertz function to the data *(21)*. The Gompertz function parameters were then used to calculate the generation time, lag phase duration, and maximum population density (log CFU per milliliter) for each strain in each growth medium.

Attachment and survival on apple surfaces and resistance to H_2O_2 wash treatments. Individual strains were grown in 10 ml of TSB at 37°C for 8 h and used to inoculate 1 liter of TSB at 0.01% (vol/vol) concentration, which was then incubated at 37°C for 16 h with shaking at 75 rpm. Cells were recovered by centrifugation at 11,170 × *g* for 10 min at 4°C, washed once with 250 ml of PW, and suspended in 2 liters of sterile distilled water. This cell suspension was used to inoculate apples. Initial cell populations were determined by serially diluting the culture in PW and surface plating duplicate 0.1-ml aliquots on TSA.

Unwaxed Golden Delicious apples (32 per experiment) obtained from a single Washington State grower were removed from storage at 4°C and inoculated in less than 1 h with the strain under investigation by submerging apples (six at once) in 2 liters of inoculum at 20°C for 5 min. The apples were placed on their sides to allow for drainage from the stem and calyx areas and left to air dry for 2 h on absorbent paper on the bench top. At this point, half of the apples (n = 16) were stored at 4°C for 24 h. The remaining apples were divided into two sets of eight. The first set was divided into duplicate composite sets of four apples. Each apple was weighed and cut into quarters using a sterile knife and cutting board. Apple pieces of each composite set were placed in a 4-liter stainless steel blender (Waring, Torrington, Conn.) with a volume of PW equal to the apple weight and blended on low speed for 1 min. The resulting blend was filtered through a filter stomacher bag (Spiral Biotech, Bethesda, Md.), and duplicate 10-ml volumes were transferred to sterile tubes. This procedure was repeated with the second composite set of fruit. The remaining eight apples were separated into two sets of four apples, and each set of apples was washed with 5% (vol/vol) H_2O_2 solution at room temperature (prepared from a 30% [vol/vol] H_2O_2 solution; Fisher Scientific, Pittsburgh, Pa.) with shaking for 1 min, briefly drained, and immediately blended. The entire procedure was repeated for apples stored for 24 h. Filtrates were diluted in PW as necessary and surface plated on the appropriate enumeration agar medium. Uninjured cells of nonpathogenic *E. coli*, *E. coli* O157:H7, and *Salmonella* were enumerated on selective MacConkey agar (Difco, Becton Dickinson), sorbitol MacConkey agar (Difco, Becton Dickinson), and xylose lysine Tergitol 4 agar (Difco, Becton Dickinson), respectively. Recovery medium (TSA) was used for enumeration of injured *E. coli* and *Salmonella* cells. After inoculation, TSA plates were incubated at 37°C for 2 h to allow recovery of injured cells and were then overlaid with the appropriate selective medium *(10)*. All plates were incubated overnight at 37°C, and resultant colonies were manually counted.

Thermal inactivation. The D-value (time to reduce bacterial populations by 1 log or 90%) of each organism under investigation was determined at 60°C ($D_{60°C}$). Individual strains were grown in 10 ml of TSB at 37°C for 8 h with shaking at 75 rpm and were used to inoculate 30 ml of each of the five growth media (TSB, TSB+G, TSB−G, TSB−GHCl, and BHI) at 0.01% (vol/vol) concentration. Cells were grown for 16 h with shaking at 75 rpm at 37°C, recovered by centrifugation, washed once with PW, and suspended in 30 ml of sterile PW. Samples with similar cell densities were heat treated at 60°C by the method of Cole and Jones *(7)*, using a Techne submerged-coil heating apparatus model tempette TE-8D (Protocol Instruments Ltd., West Byfleet, UK). Each thermal inactivation procedure was performed in triplicate. Heat-treated samples were serially diluted in PW, and duplicate 0.1-ml aliquots were surface plated on TSA. Plates were incubated overnight at 37°C prior to counting. The D-value was calculated by plotting log number of survivors against time and obtaining the reciprocal of the slope of the line using Excel spreadsheet software (Microsoft Corporation, Redmond, Wash.).

Statistical analyses. Analyses of variance with individual contrasts and Bonferroni t tests were performed to determine significant differences between population means in response to strain and medium and to determine medium-strain interactions. Comparisons of final pH values, growth parameters, attachment and resistance to H_2O_2 wash, and thermal inactivation for each strain in each medium were made using the Bonferroni least significant difference separation technique. All statistical analyses and calculations of means and standard deviations were performed using SAS/STAT software (SAS Institute Inc., Cary, N.C.).

RESULTS

Nonpathogenicity confirmatory tests. All nonpathogenic *E. coli* strains included in the study were tested for the presence of virulence genes associated with *E. coli* O157:H7. All nonpathogenic *E. coli* strains were negative for all virulence genes tested, with the exception of strain NRRL B-2783, which was positive for the O157 gene. Consequently, this strain and the O157:H43 strain were not considered as potential surrogates in the final analysis.

Changes in broth pH following microbial growth. The pH values of uninoculated broths incubated as controls decreased by 0.04 to 0.11 units following incubation overnight at 37°C. Every strain, unless otherwise noted, after being grown in broth exhibited the following relationship of final pH: TSB−G > BHI = TSB > TSB−GHCl > TSB+G ($P < 0.05$) (Table 2). An exception to this pattern was seen with *E. coli* NRRL B-14573, where a lower pH was obtained after growth in TSB−GHCl compared with growth in TSB+G, and no difference was observed in final pH between TSB and TSB+G (data not shown). No significant difference in final pH ($P < 0.05$) was noted for TSB−GHCl and BHI after growth of *E. coli* ECRC 96.0509 (data not shown). For *Salmonella* Poona, there was no difference in the final pH ($P < 0.05$) after growth in BHI, TSB, and TSB−GHCl, whereas for *Salmonella* Montevideo growth in BHI resulted in a significantly higher final pH ($P < 0.05$) than growth in TSB and TSB−GHCl, whose final pH did not differ significantly ($P < 0.05$) (Table 2).

Table 2 lists the potential surrogate strains with final

TABLE 2. *Surrogate strains with significantly different ($P < 0.05$) final pH values compared with pathogenic strains*[a]

Pathogenic strain	Growth medium: BHI		TSB		TSB+G		TSB−G		TSB−GHCl	
	Surrogate strain	pH[b]	Surrogate strain	pH	Surrogate strain	pH	Surrogate strain	pH	Surrogate strain	pH
E. coli O157:H7 SEA 13B88	96.0509[c]	6.00 ± 0.03	NS[d]	5.97 ± 0.05	B-14573[e]	4.77 ± 0.01	B-14573[e]	7.10 ± 0.03	NS	5.45 ± 0.02
E. coli O157:H7 OK	96.0509[c]	6.00 ± 0.04	NS	5.96 ± 0.03	B-14573[e]	4.74 ± 0.02	NS	7.09 ± 0.03	NS	5.41 ± 0.03
Salmonella Montevideo G4639	SL[f]	6.56 ± 0.86	B-14573[e] 96.0509[c]	6.08 ± 0.06	B-14573[e]	4.70 ± 0.06	25253[c]	7.35 ± 0.04	SL	5.93 ± 0.06
Salmonella Poona RM 2350	96.0509[c]	5.98 ± 0.04	B-14573[e]	6.07 ± 0.06	B-14573[e]	4.71 ± 0.02	NS	7.35 ± 0.02	SL	5.86 ± 0.06

[a] All *E. coli* strains, identified by strain numbers (see Table 1).
[b] Mean ± standard deviation.
[c] Significantly lower final pH ($P < 0.05$) than that of pathogenic strain.
[d] NS, no significant difference ($P < 0.05$) in final pH values between surrogate strains tested (Table 1) and the pathogenic strain.
[e] Significantly higher final pH ($P < 0.05$) than that of pathogenic strain.
[f] SL, all surrogate strains tested (Table 1) had significantly lower final pH ($P < 0.05$) than that of the pathogenic strain.

pH values significantly different ($P < 0.05$) from those of pathogenic strains following growth in each medium. Little separation was evident, with some exceptions. Both *Salmonella* strains had significantly higher final pH values ($P < 0.05$) than did *E. coli* O157:H7 strains in TSB−GHCl. All surrogate strains tested had a significantly lower final pH ($P < 0.05$) than that of *Salmonella* Montevideo G4639 after growth in BHI or TSB−GHCl. All surrogate strains tested had a significantly lower final pH ($P < 0.05$) than that of *Salmonella* Poona RM 2350 after growth in TSB−GHCl. The final pH of all surrogate strains grown in TSB−G was not significantly different ($P < 0.05$) from those of *Salmonella* Poona RM 2350. *E. coli* NRRL B-14573 grown in TSB or TSB+G had significantly lower and higher ($P < 0.05$) final pH, respectively, compared with both *Salmonella* strains. *E. coli* ECRC 96.0509 had a significantly lower final pH ($P < 0.05$) than did *Salmonella* Montevideo G4639 after growth in TSB and *Salmonella* Poona RM 2350 after growth in BHI. *E. coli* ATCC 25253 had a significantly lower final pH ($P < 0.05$) than did *Salmonella* Montevideo G4639 after growth in TSB−G. The final pH of both *E. coli* O157:H7 strains was not significantly different from that of the other microorganisms tested, with the following exceptions: *E. coli* ECRC 96.0509 had a significantly lower final pH ($P < 0.05$) in BHI and *E. coli* NRRL B-14573 had a significantly higher final pH ($P < 0.05$) in TSB+G. The latter strain also had a significantly higher final pH ($P < 0.05$) after growth in TSB−G than did *E. coli* O157:H7 SEA 13B88.

Growth characteristics. The analysis of three growth parameters (generation time, lag phase duration, and maximum population) was performed to determine the effects of strains and growth media. Generation times for the potential surrogate strains that were significantly different ($P < 0.05$) from pathogenic strains are shown in Table 3. Overall, there was no significant difference in generation times for all strains grown in BHI, TSB, TSB+G, and TSB−G, with the exception of *E. coli* NRRL B-14573 grown in BHI or TSB−G, which had a significant longer generation time ($P < 0.05$). Significantly longer generation times ($P < 0.05$) were seen when strains were grown in TSB−GHCl than when they were grown in the other broths tested, with the exception of *E. coli* ECRC 99.1232. Overall, *E. coli* ECRC 99.0512 grown in TSB−GHCl had the shortest generation time (0.09 h) among strains and media tested ($P < 0.05$). There was no significant difference in lag phase duration between the strains in any of the media (range, 1.56 to 2.55 h; data not shown). Significantly longer lag phases ($P < 0.05$) were seen when strains were grown in TSB−GHCl than when they were grown in the other broths tested (data not shown). Overall, the highest and lowest maximum population densities of all bacterial strains tested were obtained following growth in BHI and TSB and in TSB−GHCl, respectively (data not shown). Exceptions were the *E. coli* NRRL B-2783, NRRL B-3704, and NRRL B-766 strains, where the maximum population densities were obtained following growth in TSB−GHCl (data not shown). These population densities were not significantly

TABLE 3. *Surrogate strains with significantly different (P < 0.05) generation times compared with pathogenic strains grown in different growth media*

Strain	Generation time (h)[a]				
	BHI	TSB	TSB+G	TSB−G	TSB−GHCl
Pathogenic					
E. coli O157:H7 SEA 13B88	0.24 ± 0.02 A	0.25 ± 0.04 A	0.25 ± 0.03 A	0.24 ± 0.02 A	0.47 ± 0.09 A
E. coli O157:H7 OK	0.21 ± 0.04 A	0.24 ± 0.02 A	0.24 ± 0.03 A	0.21 ± 0.04 A	0.48 ± 0.02 A
Salmonella Montevideo G4639	0.28 ± 0.04 A	0.27 ± 0.02 A	0.17 ± 0.03 A	0.28 ± 0.04 A	0.49 ± 0.05 A
Salmonella Poona RM 2350	0.23 ± 0.03 A	0.21 ± 0.03 A	0.21 ± 0.02 A	0.23 ± 0.03 A	0.41 ± 0.08 A
Surrogate[b]					
E. coli ATCC 25253	NS[c]	NS	NS	NS	0.62 ± 0.02 B
E. coli NRRL B-3704	NS	NS	NS	NS	0.57 ± 0.05 B
E. coli NRRL B-14573	0.43 ± 0.03 B	NS	NS	0.43 ± 0.03 B	0.64 ± 0.07 B
E. coli ECRC 99.0512	NS	NS	NS	NS	0.09 ± 0.11 C
E. coli ECRC 99.1232	NS	NS	NS	NS	0.27 ± 0.04 D

[a] Each value represents the mean ± standard deviation of three trials. Means within each column with the same letter are not significantly different ($P < 0.05$).

[b] Surrogate strains not listed (see Table 1) showed no significant difference ($P > 0.05$) in generation time as compared with the pathogenic strains.

[c] NS, data not shown because values were not significantly different ($P > 0.05$) from generation times for the pathogenic strains.

different ($P < 0.05$) from each other nor was there any difference among the maximum population densities achieved in the other growth broths (data not shown). Overall, the growth characteristics of the pathogens were not significantly different from each other or, with few exceptions, from those of the potential surrogates.

Attachment and survival on apple surfaces and resistance to H_2O_2 wash treatments. Cell counts from treated apples were determined on recovery and selective media. Cell counts on recovery media were generally higher than counts on selective media, although significantly so only for potential surrogate *E. coli* strains ATCC 11775, ATCC 35695, NRRL B-14573, NRRL B-3054, NRRL B-3704, NRRL B-766, and ECRC 99.1232 and *Salmonella* Poona RM 2350 and *Salmonella* Montevideo G4639 ($P < 0.05$).

Table 4 lists the potential surrogate strains that had equivalent or greater attachment and survival ($P < 0.05$) on apple surfaces than did each pathogenic strain, with and without the H_2O_2 wash treatment, on day 0 and day 1. There were significant interactions between the days on which survivors were enumerated and whether or not the apples were treated with the 5% H_2O_2 wash ($P < 0.05$). Overall, samples from treated and untreated apples showed significantly higher counts on day 0 than on day 1 ($P < 0.05$). Reductions in cell numbers following 5% H_2O_2 wash treatment were only significant ($P < 0.05$) for apples sampled on day 0.

Thermal inactivation. Survivor curves demonstrated a linear decrease in cell numbers with time during heating at 60°C (curves not shown). Table 5 lists potential surrogate strains that had equivalent or greater $D_{60°C}$-values ($P < 0.05$) than did pathogenic strains. There was a significant interaction between the strains tested and the growth medium used ($P < 0.05$). The $D_{60°C}$-value of *E. coli* O157:H7 SEA 13B88 after growth in TSB+G was significantly higher ($P < 0.05$) than values obtained following growth in other growth media tested. Both *E. coli* O157:H7 strains were generally more thermal resistant than *Salmonella* strains ($P < 0.05$). However, there were two exceptions to this trend; following growth in TSB−G and in TSB−GHCl, *Salmonella* Montevideo G4639 did not significantly differ from *E. coli* O157:H7 SEA 13B88 ($P < 0.05$).

DISCUSSION

This study was a first attempt to match the growth characteristics, thermal resistance, attachment to produce, and resistance to a sanitizer wash treatment of nonpathogenic surrogate organisms to those of pathogenic counterparts. The lag phase duration observed for *E. coli* O157:H7 SEA 13B88 was considerably (28.6-fold) shorter than that reported by Whiting and Golden *(22)* for the same strain. These researchers grew the culture at 15°C in BHI containing 1.5% NaCl (pH 5.3), which could explain the longer lag phase duration. The final pH values for *E. coli* O157:H7 SEA 13B88 after growth in TSB+G and TSB−G (Table 2) were in agreement with those previously reported by Buchanan and Edelson *(6)*. Investigation of growth characteristics and final pH at stationary phase gave an indication of the metabolic behavior of each potential surrogate in each broth compared with the pathogenic strains. Very few differences were seen, which was not surprising given that all the strains tested are members of the *Enterobactericeae* and as such have similar responses to such test conditions, which are not inherently stressful. Therefore, any of the nonpathogenic *E. coli* strains tested had the potential to act as a surrogate organism for pathogenic strains tested with respect to growth characteristics and final pH value under these conditions. Considerably more separation among strains was seen following investigation of attach-

TABLE 4. *Surrogate strains that demonstrate attachment and survival equivalent to or greater than that of the pathogenic strains ($P < 0.05$) with and without the 5% H_2O_2 washing treatment on days 0 and 1*[a]

Pathogenic strain	Day 0		Day 1	
	Control	Washed	Control	Washed
E. coli O157:H7 SEA 13B88	(4.67 ± 0.66) A B-14573, 25253, 25922, B-2783,[b] 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232, O157:H43[b]	(3.15 ± 0.29) B 11775, B-14573, 25253, 25922, B-2783, B-3054, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152,[c] 97.0190, 99.0512, 99.1232, O157:H43	(4.28 ± 0.41) A B-14573, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232	(2.89 ± 0.62) B 11775, B-14573,[c] 25253, 25922, B-2783, B-3054, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152,[c] 97.0190, 99.0512, 99.1232
E. coli O157:H7 OK	(4.70 ± 0.48) A B-14573, 25253, B-2783, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232, O157:H43	(3.52 ± 0.26) B B-14573, 25253, B-2783, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512	(3.73 ± 0.30) B B-14573, 25253, 25922, B-2783, 96.0509, 97.0147, 97.0152,[c] 97.0190, 99.0512, 99.1232	(3.07 ± 0.42) B B-14573,[c] 25253, 25922, B-2783, B-3054, 35695, B-3704, B-766, 97.0147, 97.0152,[c] 97.0190, 99.0512, 99.1232
Salmonella Montevideo G4639	(4.89 ± 0.28) A B-14573, 25253, B-2783, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, O157:H43	(3.60 ± 0.54) B B-14573, 25253, B-2783, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512	(3.80 ± 0.60) B B-14573, 25253, 25922, B-2783, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232	(3.28 ± 0.79) B B-14573, 25922, B-766, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232
Salmonella Poona RM 2350	(4.87 ± 0.31) A B-14573, 25253, B-2783, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, O157:H43	(3.71 ± 0.65) B B-2783, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512	(3.38 ± 0.75) BC B-14573,[c] 25253, 25922, B-2783, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152,[c] 97.0190,[c] 99.0512,[c] 99.1232	(2.66 ± 0.84) C 11775, B-14573, 25253, 25922, B-2783, B-3054, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152,[c] 97.0190,[c] 99.0512,[c] 99.1232

[a] All *E. coli* strains, identified by strain numbers (see Table 1). Values in parentheses are the mean ± standard deviation (log CFU per gram) at each sampling point. Means within same row with the same letter are not significantly different ($P > 0.05$).

[b] *E. coli* NRRL B-2783 contains the O157 gene, and O157:H43 strains were not considered potential surrogates. These strains are included for comparison purposes only.

[c] Strains that had significantly greater ($P < 0.05$) attachment and survival than did the pathogenic strain.

TABLE 5. *Surrogate strains that demonstrate heat resistance ($D_{60°C}$) equivalent to or greater than that of the pathogenic strains*[a]

Pathogenic strain	Growth medium				
	BHI	TSB	TSB+G	TSB−G	TSB−GHCl
E. coli O157:H7 SEA 13B88	(65.99 ± 9.20)	(72.10 ± 2.62)	(90.07 ± 4.01)	(57.94 ± 12.40)	(58.72 ± 5.90)
	B-14573,[b] 25922, B-2783,[b,c] B-3054, 35695,[b] B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232[b] 157:H43[c]	B-14573, B-2783,[b] B-3054, 35695,[b] B-3704, B-766, 96.0509, 97.0147,[b] 97.0152, 97.0190, 99.0512, 99.1232, O157:H43	35695, 96.0509, 97.0152, 97.0190	B-14573, B-2783, B-3054, 35695, B-3704, B-766, 96.0509, 97.0147,[b] 97.0152, 97.0190, 99.0512, 99.1232, O157:43	B-14573, B-2783, B-3054, 35695, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232
E. coli O157:H7 OK	(72.89 ± 11.62)	(75.21 ± 13.67)	(67.82 ± 13.24)	(73.63 ± 9.97)	(68.22 ± 8.91)
	B-14573, B-2783,[b] B-3054, 35695,[b] B-766, 97.0147, 97.0152, 99.0512, 99.1232[b]	B-14573, B-2783,[b] B-3054, 35695,[b] B-766, 96.0509, 97.0147,[b] 97.0152, 97.0190, 99.0512, 99.1232	B-14573, 25922, B-2783, B-3054, 35695,[b] B-3704, B-766, 96.0509, 970147, 97.0152,[b] 97.0190, 99.0512, 99.1232	B-2783, 35695, 96.0509, 97.0147, 97.0152, 99.0512, 99.1232	B-14573, B-2783, B-3054, 35695, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.132
Salmonella Montevideo G4639	(35.14 ± 6.48)	(46.50 ± 6.64)	(38.19 ± 3.77)	(47.11 ± 73)	(40.83 ± 3.50)
	11775, B-14573,[b] 25253, 25922, B-2783,[b] B-3054, 35695,[b] B-3704, B-766,[b] 96.0509, 97.0147,[b] 97.0152,[b] 97.0190, 99.0512, 99.1232,[b] O157:H43	11775, B-14573,[b] 25253, 25922, B-2783[b] B-3054, 35695,[b] B-3704, B-766, 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190, 99.0512, 99.1232,[b] O157:H43	11775, B-14573, 25253, 25922, B-2783, B-3054, 35695,[b] B-3704,[b] B-766,[b] 96.0509,[b] 97.0147, 97.0152, 97.0190,[b] 99.0512, 99.1232, O157:H43	B-14573, 25922, B-2783,[b] B-3054, 35695,[b] B-3704, B-766, 96.0509, 97.0147,[b] 97.0152,[b] 97.0190, 99.0512,[b] 99.1232,[b] O157:H43	11775, B-14573, 25253, 25922, B-2783[b] B-3054, 35695, B-3704, B-766,[b] 96.0509, 97.0147,[b] 97.0152,[b] 97.0190, 99.0512,[b] 99.1232, O157:H43
Salmonella Poona RM 2350	(23.37 ± 2.12)	(24.51 ± 3.24)	(26.70 ± 2.91)	(23.54 ± 6.12)	(24.01 ± 1.99)
	11775, B-14573,[b] 25253, 25922,[b] B-2783,[b] B-3054,[b] 35695,[b] B-3704,[b] B-766,[b] 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43[b]	11775, B-14573,[b] 25253, 25922,[b] B-2783,[b] B-3054,[b] 35695,[b] B-3704,[b] B-766,[b] 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43[b]	11775, B-14573,[b] 25253, 25922,[b] B-2783[b] B-3054, 35695,[b] B-3704,[b] B-766,[b] 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43	11775, B-14537,[b] 25253, 25922, B-2783[b] B-3054,[b] 35695,[b] B-3704,[b] B-766, 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43[b]	11775, B-14573,[b] 25253, 25922, B-2783,[b] B-3054,[b] 35695,[b] B-3704, B-766,[b] 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43

[a] All *E. coli* strains, identified by strain numbers (see Table 1). Values in parentheses are the mean ± standard deviation $D_{60°C}$ (in seconds) for each pathogen in each growth medium.
[b] Strains that had significantly greater ($P < 0.05$) heat resistance than did the pathogenic strain.
[c] *E. coli* NRRL B-2783 contains the O157 gene, and O157:H43 strains were not considered potential surrogates. These strains are included for comparison purposes only.

ment and survival on apple surfaces, resistance to H_2O_2 wash treatment with and without storage at 4°C, growth on selective media, and thermal resistance, all situations where the strains were exposed to stressful challenges. Several strains had attachment and thermal resistance profiles similar to those seen with *E. coli* O157:H7 and *Salmonella* and would, therefore, be suitable surrogate organisms for these pathogens under such test conditions. The phenomenon of different strains of a particular organism having significantly different responses to the same stress has been reported before. Thermal resistance at 55 or 60°C of 17 different *E. coli* O157:H7 strains grown under same conditions varied significantly *(22)*. Buchanan and Edelson *(6)* found different thermal resistance profiles for three different strains of *E. coli* O157:H7 heated at 58°C following growth in TSB+G and TSB−G. Thermal resistance values of *E. coli* O157:H7 SEA 13B88 in the present study following growth in TSB+G or TSB−G were 3.7- and 2.6-fold lower than those values previously reported by Buchanan and Edelson *(6)* for the same strain grown under similar conditions. These researchers determined thermal resistance at 58°C in BHI whereas we used 60°C in PW in this study. Although thermal resistance at 60°C would be lower than that at 58°C, BHI contains more solids than does PW, which could offer protection to the cells and thus result in an increase in thermal resistance *(2)*. Sapers et al. *(19)* applied sanitizing agents, including H_2O_2, to apples inoculated with three different generic *E. coli* strains and one *Enterobacter* strain, and found significant variations in strain responses. These data further underline the necessity to be careful in the choice of nonpathogenic surrogate organisms.

There was a significant synergistic interaction between storage time and treatment (cell populations on apples decreased following storage at 4°C), reiterating the bactericidal effects of storing potentially contaminated fruit at refrigeration temperatures *(1, 15)*. Also, survivors were more resistant to H_2O_2 wash treatment following storage at 4°C (Table 4), which could be due to biofilm formation *(4)*, physiologic adaptation to stress, or survival of stress-resistant subpopulations of the organism. Although the H_2O_2 treatment significantly reduced bacterial numbers, the mean reduction was only in the range of 1 log CFU. Reduction in this range may have been achievable simply by rinsing the fruit in water, although this treatment was not included in the present study. Sapers et al. *(17, 18)* reported 1- to 2-log reductions with comparable H_2O_2 washing treatments and found greater reductions with H_2O_2 washes applied at higher temperatures (50 to 60°C).

Potential surrogate organisms for *E. coli* O157:H7 and *Salmonella* emerging from these studies include *E. coli* B-14573, ECRC 97.0147, ECRC 97.0152, ECRC 97.0190, and ECRC 99.0512 strains. These strains do not differ significantly from the pathogenic strains in their counts on either recovery or selective media (data not shown), and attachment counts were equivalent or higher than those obtained for the *E. coli* O157:H7 and *Salmonella* strains (Table 4). Further examination of the data reveals other examples of potential surrogates that differ from *E. coli* O157:H7 and *Salmonella* strains in only one or two areas and thus may be useful in certain specific situations.

Data presented here indicate that TSB and BHI are the most appropriate growth media. Overall, strains exhibited the shortest generation time, highest maximum population density, and shortest lag phase duration in TSB and in BHI. The highest thermal resistance overall was recorded for strains grown in TSB, although there are some interstrain differences, e.g., *E. coli* O157:H7 SEA 13B88 had significantly higher thermal resistance following growth in TSB+G than following growth in any other medium. *D*-values at 58°C for three different *E. coli* O157:H7 strains following growth in TSB+G were significantly higher than those following growth in TSB−G *(6)*. Similarly, *D*-values at 60°C for *Pediococcus* sp. cells grown in TSB (containing 0.5% glucose) were significantly higher than those for cells grown in tryptone glucose yeast medium (containing 0.1% glucose) *(2, 3)*. However, thermal resistance following growth in standard formulation TSB was not evaluated in these studies. Although growing the cells in TSB+G as an acid adaptation procedure may cause cell injury *(6, 14)*, in a previous study *(5)* a large proportion of such acid-adapted cells (50 to 90%) were stressed during growth to stationary phase. Such stress was likely insufficient to affect growth in generally supportive environments, as can be seen from the growth data for strains in the present study. However, the production of excess acid during glucose metabolism may have decreased thermal resistance of surviving cells to heating at 60°C, particularly for nonpathogenic strains, which do not typically have the high acid resistance reported for *E. coli* O157:H7 *(6)*. However, Annous et al. *(3)* reported that the decrease in pH of growth medium was merely indicative of glucose metabolism and was not correlated with the change in thermal resistance of *Pediococcus* sp. Similarly, in the current study growing cells in the absence of glucose (TSB−G) did not adversely affect growth rates under conditions where no other stresses were present, but the resultant cells were significantly less heat resistant than were cells grown in TSB (containing 0.25% glucose). The relatively low pH of the TSB−GHCl probably led to acid shock of the cells rather than the acid adaptation effect that cells grown in TSB, TSB+G, and BHI encounter *(3, 16)*. Cells added to TSB−GHCl were probably injured during growth in this medium, as indicated by the significantly retarded growth rates, and thus were probably less resistant to subsequent heat treatment. These results further underline the necessity for the development of a well-balanced (universal) growth medium suitable for determinations of thermal *D*-values and other growth characteristics *(2, 3)*.

Overall, higher cell counts were achieved with recovery media than with selective media as has been reported previously *(14)*, although this effect was not significant for all strains. There were no differences in counts among five of the O55 strains and the *E. coli* O157:H7 strains on either recovery or selective media, underlining the relative resistance of these strains to the stresses applied in this study and the suitability of the O55 strains as surrogates for *E. coli* O157:H7.

E. coli ECRC 97.0152 was more similar to the *E. coli* O157:H7 strains than any other strain tested, although any of these O55 strains probably would be a good surrogate for *E. coli* O157:H7. Clonal relationship studies have established that the O157:H7 serotype is closely related to a group of O55 strains associated with infantile diarrhea *(23)*. The O157:H7 serotype most likely arose from an O55:H7-like ancestor through genetic recombination events that added virulence genes to a nonvirulent *E. coli* genome *(23)*. This scenario may explain the close correlation between the observed characteristics of the O55 strains and those of the O157:H7 strains in the present study.

Thermal resistance profiles observed in this study largely agreed with those reported in the literature *(6, 9, 22)* and were the only area in this study where a large degree of separation was observed between *E. coli* O157:H7 and *Salmonella* strains. Any one of the O55 strains mentioned could be a good surrogate organism to use in studies involving commodities, such as melons, that may be contaminated with both *Salmonella* and *E. coli* O157:H7 and that do not readily demonstrate thermal damage *(1)*. Potential surrogate strains with equivalent or higher thermal resistance than pathogenic strains in all growth media tested and thus were considered potential surrogates for these pathogens based on this criterion include *E. coli* ECRC 97.0152, ECRC 97.0147, and ATCC 35695. Other potential surrogate strains that did not differ significantly from pathogenic strains and may be useful in certain specific situations are listed in Table 5. However, although there is some merit in erring on the side of caution by using a surrogate strain with higher thermal resistance than the target pathogenic strain (in this case, *Salmonella*), the use of such a strain could ultimately lead to unnecessary waste of heat energy and overprocessing of the target food product, with associated sensory damage, particularly if there is low likelihood of the presence of an organism as heat resistant as *E. coli* O157:H7. Therefore, difficulty arises when trying to recommend a good surrogate for *Salmonella* from the strains tested. Although *Salmonella* strains tested were among the lowest in terms of thermal resistance in this study, they had relatively high rates of attachment and survival, and very few of the nonpathogenic strains tested had the same resistance patterns. *E. coli* NRRL B-766, for example, was similar in terms of both attachment and heat resistance to *Salmonella* grown in TSB and TSB−G, but employment of this strain would significantly overestimate the heat resistance of *Salmonella* in the other growth media tested. Sensitivity to heat should be of great importance for selecting a surrogate strain in view of the limited efficacy of sanitizing washes and the success of surface pasteurization with hot water *(1, 4, 17)* or steam *(13, 20)*. Although, surface pasteurization resulted in significant improvement in microbiological qualities of some fruits and vegetables and improved the shelf life of cantaloupes *(1)*, it caused thermal injuries to apples *(17)*. Potential surrogates that have heat resistance profiles equivalent to those of *Salmonella* Poona and *Salmonella* Montevidio strains include *E. coli* ATCC 11775, ATCC 25253, ATCC 25922, NRRL B-3054, and NRRL B-766. In this study, we found many other potential surrogates that did not differ significantly from these strains in individual growth media. Annous et al. *(1)* reported similar responses by *Salmonella* Poona RM 2350 or *E. coli* ATCC 25922 on cantaloupes to commercial-scale surface pasteurization. Thus, based on thermal characteristics *E. coli* ATCC 25922 should be an appropriate surrogate for use in evaluating the efficacy of surface pasteurization for reducing and/or eliminating *Salmonella* Poona RM 2350 on cantaloupes in a pilot plant environment. Research with additional strains of *E. coli* O157:H7 and *Salmonella* and possibly other nonpathogenic surrogates is recommended to identify a effective all-purpose surrogate organism for *Salmonella* and *E. coli* O157:H7.

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Title: *In Vitro* and *In Situ* Techniques to Examine the Role of Roots and Root Exudates During Am Fungus-Host Interactions

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IN VITRO AND *IN SITU* TECHNIQUES TO EXAMINE THE ROLE OF ROOTS AND ROOT EXUDATES DURING AM FUNGUS-HOST INTERACTIONS

Subtitle: Signaling processes between AM fungi and host roots

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Abstract Although it is believed that root exudates play a major role in the infection and colonization of hosts by arbuscular mycorrhizal (AM) fungi, the actual role or mode of action of exudates has been elucidated only recently. New developments in *in vitro* culture techniques and *in situ* compartmental analyses have allowed the role of host roots and exudates during AM fungus colonization to become clear. These recent developments also have provided a way to study the colonization of host roots in the presence of nonhost root systems and have provided a more precise way to study nonhost exudates. The value of the new techniques is that they allow specific events of the life cycle of AM fungi to be studied.

Key Words: Roots, root exudates, signaling/recognition processes, AM fungi

1. Introduction

Arbuscular mycorrhizal (AM) fungi form an obligate symbiotic association with most plants. The symbiosis is mutually beneficial since the fungus receives carbon from the host and the host receives greater vigor through increased uptake of immobile soil nutrients. The practical use of AM fungi to supplant or reduce chemical fertilization in agricultural fields has been hindered by the lack of inoculum production technologies which, to date, all require the presence of a host root.

Axenic culture of AM fungi has not been accomplished and may only be achieved with a better understanding of each step of the fungal life cycle. The life cycle of an AM fungus (Figure 1) can be represented with seven steps although only six steps appear to require recognition or communication events between the fungus and host. Some steps appear to be one way communication events while others may involve a dialogue between fungus and host root. The current knowledge about the role of exudates in AM fungus-host interactions has been gained through recent developments in *in vitro* culture techniques and *in situ* compartmental systems. The results achieved with these techniques will be emphasized here.

2. Spore Germination

Spore germination (Step 1, Figure 1) is reviewed by Giovannetti (this volume) and will be mentioned briefly. Although germination is a major step in the life cycle of an AM fungus, it can occur with the appropriate environmental conditions in the absence of a host root [8, 14, 19]. The early work of Mosse [38], showed it was possible to germinate AM fungi in axenic culture in the absence of the host. Since this report, no conclusive evidence has been presented on the effects of host roots or root exudates on spore germination [24]. Roots of nonhost plants have been reported to stimulate [51] or inhibit [62] spore germination while root extracts strongly inhibited spore germination [61]. Depending on the experimental conditions, the host plant tested, and the AM fungus used, host roots either positively affect spore germination or have no effect [24 and references therein]. Recently, the germination of *Glomus mosseae* spores in the presence of host roots or nonhost roots was neither stimulated by the host nor inhibited by the nonhost [24]. A reasonable conclusion from this information would be that AM fungi are not able to discriminate between hosts and nonhosts during spore germination events.

3. Role of Exudates During AM Fungus-host Root Interactions

Although exudates do not generally improve spore germination, they can stimulate AM fungus hyphal growth, colonization of roots, or both [6]. Increased hyphal growth both *in vitro* and *in situ* in response to root exudates or root volatiles from host plants has been reported [39, 41, 26, 10, 40, 6, 19, 32]. There is no direct evidence for the release or exudation of inhibitory compounds from living nonhost roots although several papers have suggested this possibility [50, 31]. In both papers, the exudates from nonhosts were not tested directly. Nonhosts, in general, appear to lack factors which induce hyphal growth [24 and references therein].

The early work on plant exudates was reviewed and summarized by Manorik and Belima [37]. Since this review, studies were done to determine more specifically the influence of exudates on AM fungus colonization of roots [2, 54, 27]. Quantification of exudate components was still considered more important than quality of the exudate, but no consistent quantitative differences between host and nonhost were apparent (54). The literature on methods of exudate analyses again was reviewed by Curl and Truelove [12] and although existing techniques had been modified and biochemical procedures of analysis were refined, no revolutionary methods had been devised since the late 1960's. New methods have been developed since the mid 1980's which have provided new insights into the role of exudates in AM fungus-host root interactions and these results will be emphasized.

3.1. BICOMPARTMENTAL IN VITRO CULTURE SYSTEM TO STUDY THE ROLE OF ROOT VOLATILES ON AM FUNGUS HYPHAL GROWTH

The first *in vitro* culture of vesicular-arbuscular mycorrhiza under aseptic conditions was accomplished by Mosse [39]. Although her approach did not gain much popularity, the

Canadian group at Laval University brought the *in vitro* culture approach to the forefront. A method was developed to study the influence of root volatiles and exudates [6] on AM fungal growth via a bicompartmental system. The two compartments were created by placing an uncovered small Petri dish (100 × 15 mm) into a large Petri dish (150 × 15 mm) with gelled medium in both compartments. The germinated AM fungus spore was grown in the small dish, physically separated from the roots in the large dish. Only volatile compounds from the roots could interact with the fungus. Further work indicated that one of the major volatile compounds that stimulated fungal growth was CO_2 [6].

Figure 2 shows a tracing of a similar bicompartmental system and the growth of the fungus in the presence and absence of root volatiles. Even though this test system clearly showed that volatiles from host roots can stimulate fungal growth, compounds other than CO_2 would be difficult to identify since no simple bioassay is available to monitor active volatile compounds during purification.

3.2. THE USE OF MEMBRANES TO PHYSICALLY SEPARATE GERMINATED FUNGAL SPORES FROM HOST ROOTS IN SITU

A technique developed by Giovannetti *et al.* [19, 20] has provided a method to study the interactions between host roots and AM fungi before physical contact. Roots were sandwiched between two millipore membranes (0.45 mm pore diameter) and a second millipore membrane inoculated with sporocarps of *G. mossae* was placed over the membrane sandwich containing the roots. The experimental unit then was placed in a pot of acid washed, sterile quartz grit. Host roots elicited a local change in the branching of hyphae of AM fungi and this hyphal branching was not due to physical contact with the root but to the presence of a stimulator in the exudate which passed through the membrane. From these results, they postulated that there is a concentration gradient of the chemical stimulus (which has a molecular weight of less than 500 Da, [23]) from the roots to nearby zones and the stimulus is perceived by the fungus only at high concentrations occurring at or near the root surface. They further speculated that the differential hyphal morphogenesis elicited by the root signals facilitates the search for suitable sites for hyphal adhesion and appressorium formation.

3.3. BIOASSAY FOR TESTING EXUDATES AND OTHER ROOT FRACTIONS VIA IN VITRO CULTURE TECHNIQUES

The dual *in vitro* culture system developed by Bécard and Fortin [4] allows for the observation of the interaction between a single germinated spore and a host root. A hyphal tip growing at a distance from the root will produce a few elongated lateral hyphal branches. As the hyphal tip grows closer to the root, more lateral branches are produced and they are less elongated. When a hyphal tip approaches very near the surface of a root, a three dimensional, very bushy-type pattern of short hyphal branches is induced.

An *in vitro* culture technique was recently developed that has provided a direct way to study the effects of host root or nonhost root exudate on the growth and development of AM fungi without the presence of a root [46]. Spores are germinated and grown on gelled medium and after 3 or 4 days growth, two small holes are placed about 2-3mm in

front of any growing hyphal tip. The holes are filled with 8 to 10 μL of either diluted or concentrated crude exudate produced by roots growing in liquid culture or exudate components that have been separated by various chromatographic methods. This bioassay, using various concentrations of host root exudate, duplicated the hyphal branching patterns produced by AM fungus hyphae as they approach an intact host root [48]. Differential hyphal morphogenesis can be detected *in vivo* as early as 24 hr after challenging fungal mycelium with host roots [21, 18] and within 6 hrs after hyphal tips have been exposed to root exudate *in vitro* [46].

This technique was also used to show that nonhost (sugarbeet) roots exude compounds that initially inhibit hyphal tip growth (Nagahashi, unpublished data). When the tip of a germ tube was inhibited, a lateral hypha (recovery hypha) rapidly formed and if the new tip was inhibited, another recovery hypha formed. Eventually, the inhibitor concentration dissipated by diffusion and a recovery hypha became the new dominant germ tube. These results with host and nonhost exudates *in vitro* substantiate the earlier hypothesis put forth by Giovannetti *et al.* [21] that the fungus senses a concentration gradient of compounds as it approaches a root surface.

The bioassay can be used to test soluble chemical compounds as well as particulate fractions such as root cap border cells, isolated membranes, or mucilage. Sloughed root cap cells, now referred to as border cells (BC) by Hawes *et al.* [28] and root mucilage may also contain components which affect the growth of AM fungi [21, 5, 49]. A strong correlation between AM fungal colonization and border cell production recently has been shown [49]. In general, plant families that were high in border cell production (such as Leguminosae and Gramineae) also had a propensity to be colonized by AM fungi whereas families that were low in BC production (such as Brassicaceae and Chenopodiaceae) were less likely to be mycorrhizal.

Border cells were collected from Ri T-DNA transformed carrot roots grown in liquid culture to directly test their effects on AM fungal growth (Figure 3A). The BC fraction induced hyphal branching of germinated *Gigaspora gigantea* spores (Nagahashi, unpublished data) in the bioassay. Although soluble exudate fractions contained the highest levels of hyphal branch inducers, a particulate fraction also stimulated branching. This observation has physiological relevance since BC may adhere to the surface of a root as the root continues to grow (Figure 3B) and consequently, act as a source of signal far from the major exudation site at the root tip. This source of signal could, in part, substantiate the fact that random infection events can occur along the length of a root in both immature and differentiated regions [55].

3.4. THE USE OF MULTIPLE COMPARTMENTS FOR IN SITU STUDIES

Vierheilig *et al.* [60] used a three compartment system to provide a functional assay for growth and spreading of mycorrhizal fungi in root-free soil and in the rhizosphere. Each compartment was 2 cm wide (in certain studies the center compartment was 0.5 cm wide) and the compartments were separated by 60 mm mesh nylon screens which could be penetrated by hyphae but not by roots. This system was used to determine whether a nonhost root system (lupin), imposed between the AM fungus inoculum and a host acceptor plant, could influence the spread of the fungus toward the uninoculated host.

There was no difference in the colonization of the acceptor plant compared to controls indicating that the rhizosphere of the nonhost root did not interfere with the spreading of AM fungi. It was already reported that lupin roots did not affect the germination or growth of *G. mosseae* [1, 19] however, the root exudates of lupin were reported to hinder hyphal attachment [19]. The behavior of germinated spores of *G. gigantea* in the presence of concentrated nonhost root exudate (discussed in a previous section) was consistent with these results. At high concentration (as would be the case when a fungal hypha grows close to the surface of a nonhost root), the hyphal tip is inhibited but recovery branches would allow the fungus to move around the nonhost root into areas of less concentrated inhibitor (Nagahashi, unpublished data). Thus the fungus could spread through a nonhost rhizosphere and consequently, colonize a host root.

The compartmental system also was used to determine whether AM fungus hyphal tips are attracted to host root exudates [63]. Directional growth or attraction of germ tubes toward a host root has been reported for AM fungi [41, 59, 42] although no conclusive data was shown that support directional growth. The very recent work of Vierheilig *et al.* [63] has suggested an attractional effect of bean root exudates for *G. mosseae* hyphae. A three compartment system was used in which two outside compartments (2 cm wide) containing host plants were separated from a middle compartment (0.5 cm wide) containing the inoculum. The middle compartment had a nylon filter on one side and a polytetrafluorethylene (PTFE) membrane on the other. The nylon filter allows hyphal penetration and diffusion of water soluble root exudates while the PTFE allows hyphal penetration but no exchange of water soluble exudates. They concluded that tomato root exudate did not attract hyphae of *G. mosseae* but bean root exudate did. This data can be interpreted another way by assuming tomato roots have more volatile signals (which can pass through the PTFE membrane) than bean roots and therefore, show a bigger synergistic response in the presence of water soluble exudates. This would argue against an attractional growth. Assuming their interpretation was correct, the directional attraction was not a general phenomenon since one host root showed a response while the other host root did not.

The only other evidence for attractional growth has been reported by Koske [33] where aerial hyphae of *G. gigantea* showed a chemotropic response to volatile root signals. This chemotropic response has not been demonstrated for other AM fungal species. It should be mentioned that because AM fungus hyphae respond to a concentration gradient of soluble and volatile signals, what can initially appear to be an attraction may simply be a proliferation of branches in response to a higher dose of signal. This burst of branching only occurs very near a root surface where the concentration of signal is the highest and could be misread as an attraction to the root. The *in vitro* bioassay was used to determine if a chemotropic response actually can occur. The primary germ tube or secondary hyphae never attempt to grow toward concentrated root exudates applied 1.5-2.0 cm and at right angles to a growing hyphal tip (Figure 4). Branching occurs behind the growing tip and the tip always continues in the direction it was pointing before the challenge with exudate (Figure 4). Similarly, the primary germ tube or secondary hyphae of an AM fungus never attempt to grow toward a root (we have tested Ri T-DNA transformed carrot roots, nontransformed tomato roots and roots from intact sterile corn seedlings) when grown in dual culture (Figure 4).

4. Appressorium Formation and Definition of a Functional Appressorium

4.1. DEFINITION OF AN APPRESSORIUM

The third step in the life cycle, appressorium formation, has been studied by many authors [19, 20, 17, 25, 58, 34, 9] and a recent article suggested the need for a clearer definition of an appressorium in AM fungus-root interactions [24]. Appressoria of AM fungi are infection structures that are flattened, elliptical hyphal tips which form when germinated endomycorrhizal spores or infective extradical hyphae come in contact with a host root. They also have been defined as hyphal apices, not necessarily swollen, attached to the root surface [58]. These definitions of appressorium are well within the accepted definition given in A Dictionary of Plant Pathology [30]: "An organ formed from a hypha for attachment to a host before penetration; it may be a distinct cell, sometimes with a thickened wall, or it may be undifferentiated from the parent hypha. In the latter case it is distinguishable only by its adherence to host tissue." Even though obligate parasites can form appressoria on synthetic surfaces [13], the appressorium is the first cell to cell contact event and its formation is considered to be the most important evidence indicating the successful recognition between a fungus and host [56]. The definition is straightforward although mycorrhizal researchers have been reluctant to call any such described interaction with a nonhost cell wall or nonhost root surface an actual appressorium. Furthermore, an appressorium-like structure was reported when a hyphal tip from an AM fungus interacted with a hypha from a heterologous AM fungus [19]. The appressoria reported on isolated cell walls of a host root [47] were similar to those formed on excised nonhost roots [19], heterologous AM fungus hypha [19] as well as those formed on roots of a host [35, 17, 9].

There is a major morphological difference between the appressoria formed on isolated cell walls and excised roots of senescing nonhosts when compared to those formed on host roots. Hyphal fans are often observed with intact host roots and multiple appressoria are formed. Only host roots exude branching signals and the signals are most concentrated on the surface of the host root. The hyphal fan-like structures shown by a number of researchers [9, 17, 24] may only occur near an actively exuding site and this results in multiple appressoria. It should be noted that not all appressoria formed on host roots are part of the hyphal fan-like structure. A hypha may grow a considerable distance in an epidermal groove before an appressorium is formed [35, 41, 17, 19]. This was more typical of appressoria formed on isolated host root cell walls and nonhost roots.

4.2. APPRESSORIA FORMATION ON ISOLATED CELL WALLS

It has been proposed that cellular signals (exudates or root mucilage) are necessary before appressoria of AM fungi are formed [32, 22], and the lack of signals in nonhost exudate prevents appressoria formation [19]. Topographical or biochemical signals on the root surface also are believed to be necessary for appressoria to form [22] and recently some compounds hydrolyzed from host cell walls were shown to stimulate hyphal growth [15].

To address the question of whether the formation of appressoria is governed by cellular signals (exudates or root mucilage) or topographical or biochemical signals of the cell wall, cell walls from carrot roots were isolated, purified, sterilized, and added to gelled

medium in Petri plates in the presence of a germinated AM fungus spore. The isolation technique allowed for the rapid purification of very large cell wall fragments (up to 5-6 cells long) which retain biological activity [44, 45, 47]. Commonly used cell wall isolation procedures would not be successful for these types of studies because cell wall fragments isolated with these techniques are typically less than one cell length long [11, 43] and the surface topology between epidermal cells is destroyed. Results showed that both *G. gigantea* and *G. margarita* form appressoria on isolated host epidermal cell wall pieces in the absence of exudate signals, mucilage, protoplasts, or intact cells [47]. The cell wall fragments achieved with this technique are large enough so that they could also be treated with host root exudate to see if hyphal fans can be formed on purified host cell walls.

Since appressoria were formed on isolated cell walls in the absence of exudate, it was clear that exudate signals for attachment and/or appressoria formation were not necessary. Although specific topographical signals of a host were reported for growth orientation and appressorium formation of a parasitic fungus [29], this type of study has not been done for AM fungus-host associations. The use of purified host cell walls in conjunction with *in vitro* culture techniques has opened this avenue of research so that physical as well as biochemical properties of the binding site can eventually be determined.

4.3. DEFINITION OF A FUNCTIONAL APPRESSORIA

If appressoria or appressoria-like structures are formed on nonhost cell surfaces, on other AM fungal hyphae, on host root surfaces, and on isolated cell walls of host roots, the most suitable question should be what is functional appressorium? When appressoria were formed on excised nonhost roots or isolated host cell walls, one consistent observation was the lack of penetration hyphae or penetration pegs after appressoria formation. Similarly, the appressoria formed on "early" myc-mutant plants also did not form penetration hyphae [24] and appear to be missing a signal for this step. A **functional appressorium** then would be a structure attached to the surface of a root that forms penetration hyphae. An appressorium that will not form penetration hyphae or form a penetration hypha that is rapidly septated would be nonfunctional, by this definition.

4.4. THE ROLE OF HOST EXUDATES IN APPRESSORIUM FORMATION

Several pieces of information have supported a general conclusion about the role of exudates in appressoria formation: 1. the initial contact of a fungal parasite with host surface is thought to be a random event [36] since a parasite may establish many contacts with both a host and nonhost. 2. host exudates, in general, do not attract or cause directional growth of AM fungi. 3. hyphal fans form very near the surface of a host root. In view of these results, it would appear that the role of the branching signals in the exudate would be to increase the chance of contact with a host cell wall binding site as AM fungal hyphae approach the root surface. The possibility that the increased branching has an assimilatory role [22] should also be considered.

Whether an exudate signal or a cellular signal is necessary to form penetration hyphae has not yet been determined. This experiment conceivably could be done with isolated host cell walls, germinated AM fungus spores, and the application of exudate or root extract.

5. Conclusions: Potential Signaling Events Throughout the AM Fungus Life Cycle

Spores from AM fungi can germinate under appropriate storage and environmental conditions, so no apparent host signal is necessary for the first step of the life cycle (Fig. 1). The first response of a fungus to host root is the stimulation of hyphal growth (Step 2). It is now well documented that host roots not only promote hyphal growth of AM fungi, but they also induce changes in hyphal growth pattern and morphology by inducing branching and stimulating the production of short branched hyphal fans [21]. This morphologically identifiable step is a one-way communication event since the AM fungus recognizes a component exuded by the host root. Host roots, grown *in vitro*, have never been exposed to the fungus prior to the bioassay so the hyphal branching response occurs as a result of a constitutive host signal (or signals). The hyphal contact with the surface of the host root appears to be a random event and the increased branching of a fungus as it approaches a root surface would increase the probability of contact to form an appressoria.

Appressoria formation (Step 3) is also a one-way event because this structure can be formed on isolated cell walls of host roots. No host plasma membrane-associated signal or soluble cytosolic signal is required for appressorium formation. If particular biochemical compounds and/or topological components at the host cell wall binding site are necessary for the fungus to attach, these signals are also constitutive. Appressoria can form between hyphae of heterologous AM fungi [19], on excised or senescing nonhost roots [19], as well as on host root epidermal cell walls. However, formation of functional appressoria with penetration hyphae and subsequent intraradical hyphal development (Step 4) only occur with intact host roots.

The formation of penetration hyphae and intercellular hyphal spread (intraradical growth) are considered as a single step in the AM fungus life cycle (Step 4). Formation of penetration hyphae would appear to require a third signal from the host. This signal may induce hydrolytic enzyme production in the fungus which would allow hyphal penetration of epidermal and cortical cell walls of the host. Concomitantly, the fungus must evade or not induce the host defense system so this step must be a two-way dialogue between the fungus and host. Identification of the signal (or perhaps signals) that trigger the fourth step may be the key to the successful axenic culture of an AM fungus. When the life cycle of an obligate plant parasite (*Puccinia graminis*) was completed by nutrient manipulation in the absence of a host, the fungus first had to produce a morphologically identifiable infection structure [64]. Formation of a functional appressorium with penetration hyphae may be the key step to attain before hyphae of AM fungi are able to take up and metabolize hexose (Douds *et al.* [15]) and thereby complete their life cycle in the absence of a host.

Arbuscule formation (Step 5) also is likely to be a two-way interaction event. This interface between host and fungus is believed to be the site of nutrient exchange between the symbionts [7]. Ultrastructural information and ultrastructural localization studies of arbuscules recently have been reviewed [7] and have documented the considerable modifications of the host cell wall, fungal cell wall, and host cell plasma membrane made during arbuscule formation. Although arbuscules have a unique morphology, it is worthwhile to note that arbuscule-like branched structures occur among extraradical hyphae after colonization of a host root in dual culture [41, 3]. Arbuscule-like branches are also induced when concentrated host exudate is applied directly to growing hyphal tips [48]. The

significance of these branched structures is not known but perhaps manipulation of nutrients in the presence of these structures would prove fruitful for axenic culture.

The development of extracellular mycelia or extraradical hyphae (Step 6) may occur spontaneously once exchange of nutrients between symbionts has begun. There are structural differences between the extraradical hyphae and hyphae growing from a spore [16], and the arbuscule-like structures in the extraradical hyphae only occur after colonization of the root. Whether these structures or proliferation of extraradical mycelium in general require a host signal or occur as a natural consequence of the newly acquired nutritional status remains to be answered. Similarly, the seventh and final step in the life cycle of AM fungi is sporulation which also may occur as a result of the newly acquired nutritional state.

Future research on the final two steps of the life cycle could resolve whether nutrient status, host signals, or a combination of both is necessary to complete these steps. The recently developed split plate technology of St.- Arnaud *et al.* [57] could allow one to study extraradical hyphal growth and sporulation events without first having complete knowledge of all previous steps.

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7. Figure Legends

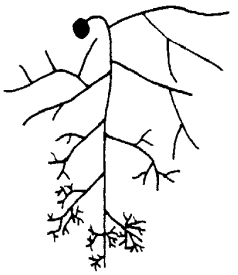
Figure 1. Schematic representation of the life cycle of an AM fungus. Some steps appear to be one-way communication events, others are two-way, and some may or may not require communication between fungus and host.

Figure 2. The *in vitro* bicompartmental system showing the growth of a germinated *Gigaspora gigantea* spore in the presence and absence of root volatiles. The germinated spore was allowed to grow for 3 days on gelled M medium in the inner compartment which was physically separated from the outer compartment. The outer compartment contained gelled M medium plus or minus Ri T-DNA transformed carrot roots. Adapted from the technique of Bécard and Piché (1986). A. Control without roots. B. With carrot roots.

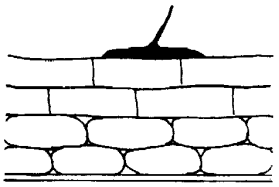
Figure 3. Micrographs of border cells associated with Ri T-DNA transformed carrot roots grown in culture. A. Border cells or root caps (arrows) were isolated from carrot roots grown in liquid culture. Insert shows a higher magnification (bar = 80 μm) of a root cap taken with a phase contrast microscope. B. Border cells adhering to the surface of carrot roots grown on gelled medium. Note the presence of sloughed border cells adhering to the root surface a considerable distance from the root tip (white arrows). Black arrow marks an area 7cm from a root tip.

Figure 4. The lack of a chemotropic or directional response of hyphae of *Gigaspora gigantea* in the presence of a host root (A) or host root exudate (B). A. Carrot roots (R) and fungus grown in dual culture. The primary germ tube or main lateral branches nearest the host root show induced branches but the tip of the germ tube or main lateral hyphae continue growing in their original direction. B. Hyphal branching, in response to carrot root exudate. Branching always occurs behind a growing tip and the tips continue to grow in their original direction. Dots indicate the site of exudate application (E) or 70% ethanol control (C). Solid traced lines indicate the growth at the time of exudate application. Broken lines indicate growth 36 hrs after treatment.

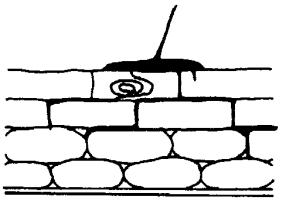
1. **GERMINATION: Plant signals are not essential for this step to occur**



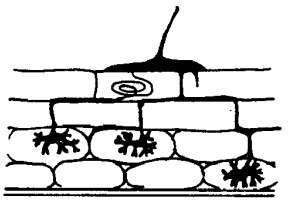
2. **HYPHAL GROWTH AND BRANCHING: A one-way communication event where the fungus senses a concentration gradient of constitutive hyphal branching stimulators exuded by a host root.**



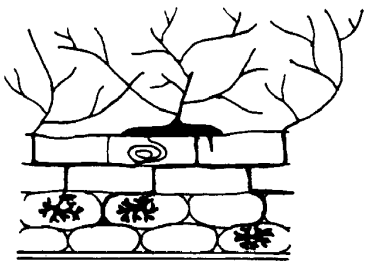
3. **APPRESSORIA FORMATION: Appears to be a one-way event and if topographical or biochemical signals are present on the cell wall, these signals are also constitutive.**



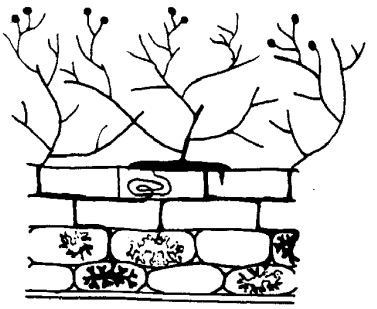
4. **PENETRATION AND INTRARADICAL GROWTH: A likely two-way communication event. Some AM fungi form coils while others spread directly to the cortical region. The fungus must evade or not turn on the host defense system.**



5. **ARBUSCULE FORMATION: A likely two-way communication event. This interface within a cortical root cell is believed to be the site of nutrient exhange between symbionts.**



6. **EXTRARADICAL HYPHAL GROWTH: May or may not require a signal from the host. Could develop as a result of the newly acquired nutritional status.**



7. **SPORULATION: May or may not require a host signal. Could also result from the newly acquired nutritional status.**

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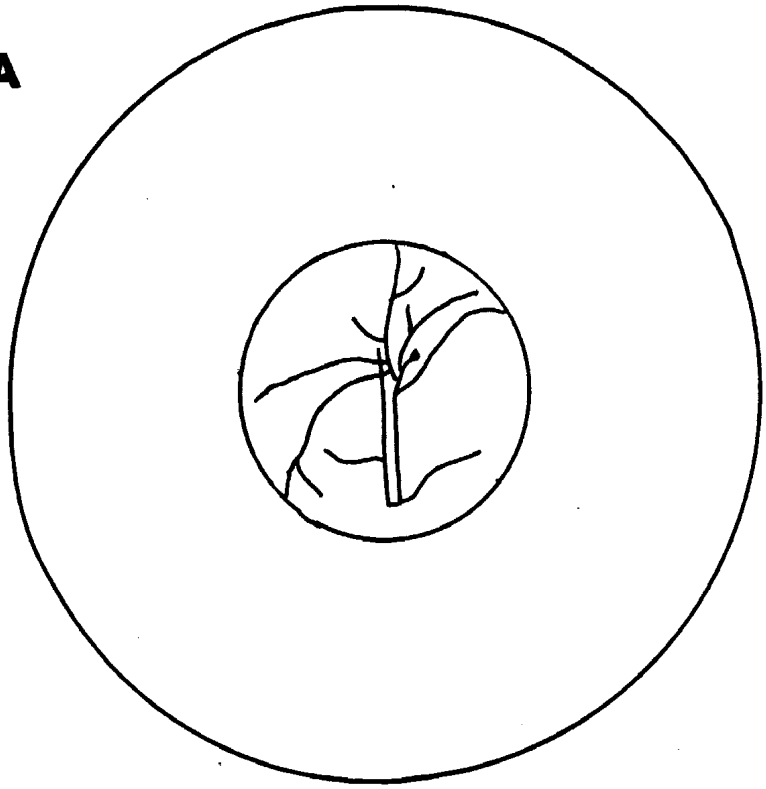

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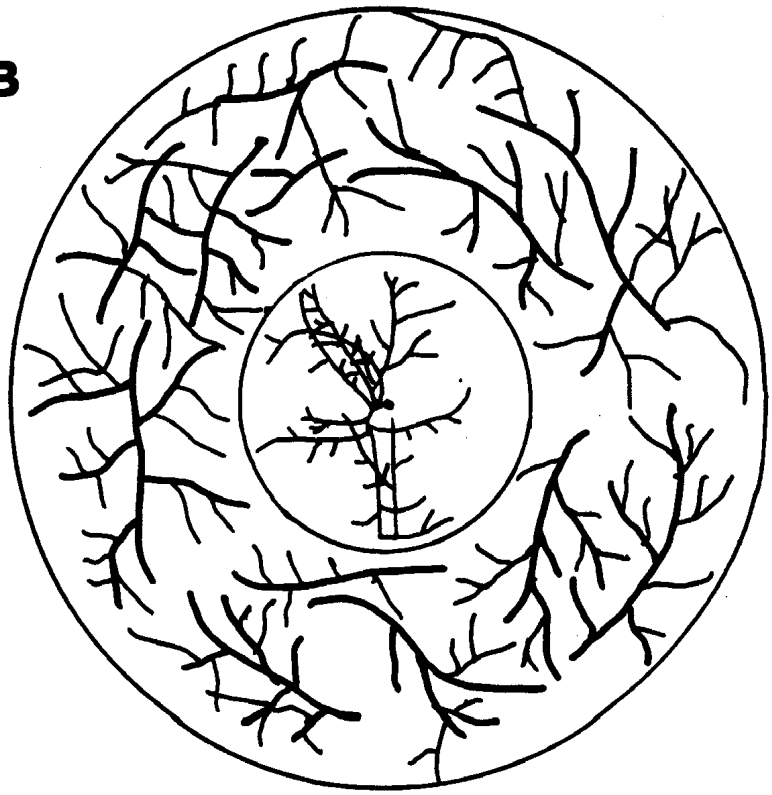

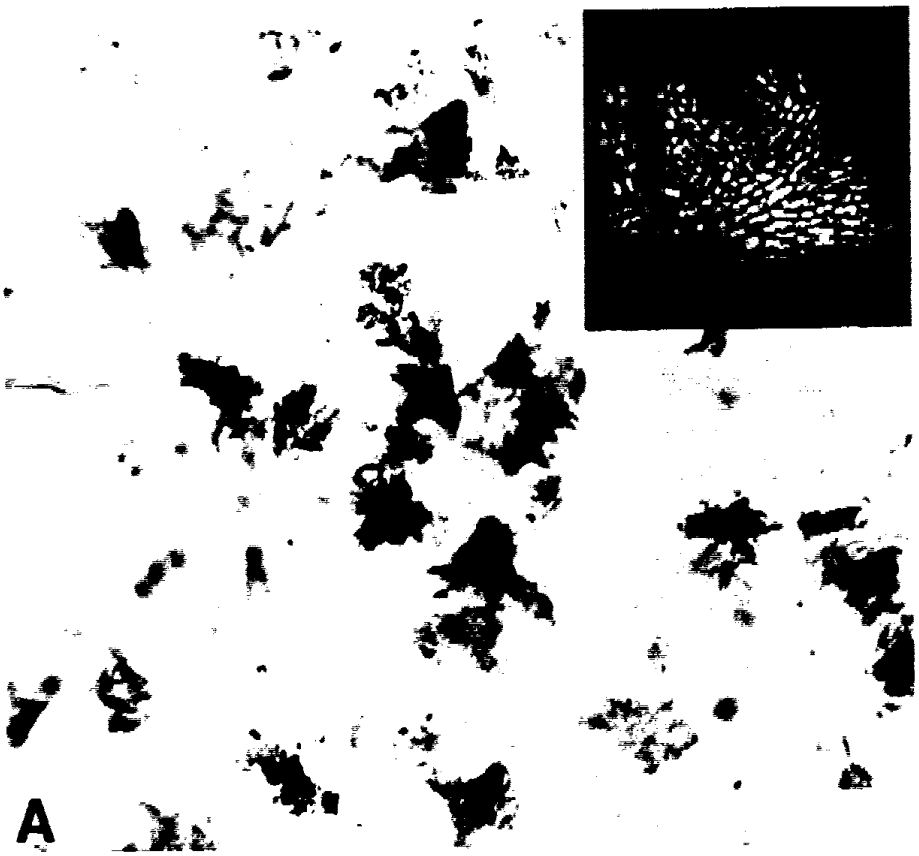
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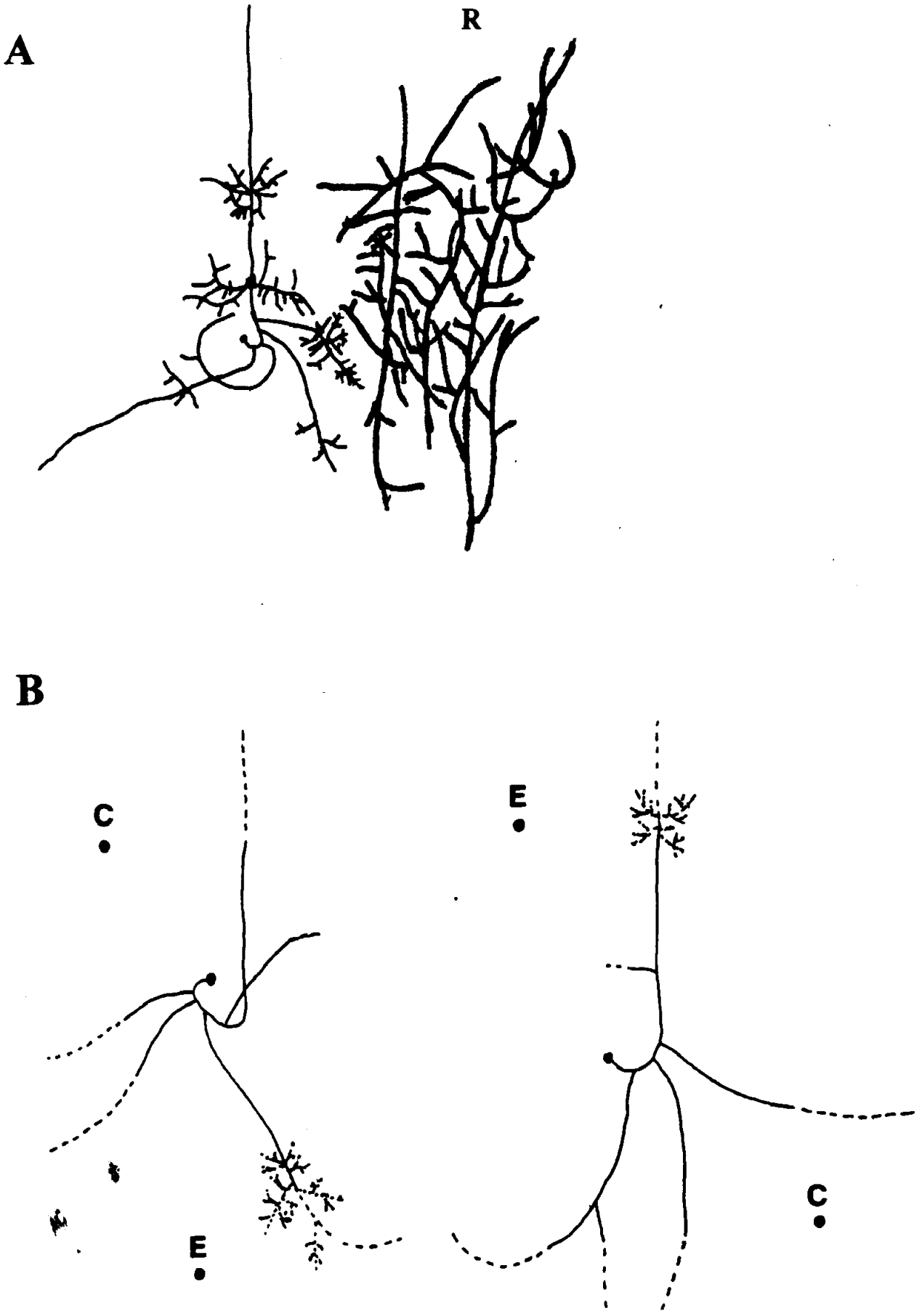
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A single sorbent for tetracycline enrichment and subsequent solid-matrix time-resolved luminescence☆

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Abstract

The aim of this study was to search for a sorbent that could act as an extraction phase and as a support for solid-matrix time-resolved luminescence (SMTRL). Four potential sorbents were investigated for this purpose using tetracycline (TC) as a model analyte. Sorbents prepared from C18 silica gel or calcium cross-linked pectin gel were able to extract TC from dilute solutions. Europium(III)–TC complex adsorbed on the surface of C18 generated the most intense TRL signal when measured at $\lambda_{ex} = 388$ nm and $\lambda_{em} = 615$ nm. This method achieved a 1 ng/ml limit of detection (LOD) with a 100 μl sample solution in a repeated spotting mode. Hyphenation of sorbent extraction and SMTRL was demonstrated using C18. This method is suitable for screening of TC in foods or aqueous solutions and can be extended to other luminescent lanthanide-chelating analytes in physiological or environmental samples.

Keywords: Time-resolved luminescence (TRL); Tetracycline; Europium; Sorbent; Pectin

1. Introduction

Tetracycline (TC, Fig. 1) is a broad-spectrum antibiotic, used in farm animals for the treatment of a wide range of bacterial infections. Although TC is generally regarded as relatively non-toxic, frequent use results in residue accumulation and antibiotic resistant bacteria. It also produces a large number of adverse effects to sensitive individuals, such as sore mouth and perineal itching, dizziness, light-headedness, diarrhoea and photosensitivity. Therefore, the allowance of TC residue in foods is regulated and routinely monitored [1,2].

TC has intrinsic molecular fluorescence. The formation of a complex with lanthanide ions, e.g., Eu(III), allows intramolecular energy transfer from excited TC to the emitting level of lanthanide ions. By funneling energy to a narrow atomic emission, fluorescence intensity is enhanced by an order of magnitude. As a result, the luminescence possesses large Stokes shifts (200–300 nm) and long excitation lifetime, so both spectroscopic and temporal discriminations can be implemented to enhance selectivity. This analytical approach is known as lanthanide chelate time-resolved luminescence (TRL).

Although TRL has successfully been used for TC determination in different matrices [3–6], sample preparation is a time consuming and costly procedure. Other concerns with complicated sample preparation steps include losses of analyte, reduced precision and potential use of hazardous solvents. This poses a big obstacle to productivity especially for laboratories that routinely analyze a large number of samples [7,8]. Efforts have been devoted to simplify analytical procedure by employing improved devices or integrating

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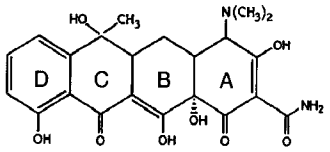

Fig. 1. Structure of tetracycline. It has been found that at neutral or lower pH, the bonding of TC with Eu(III) occurred at position 1, while at a higher pH the chelate bonding occurred at position 2 [9].

separation and spectroscopic steps into one system. For example, lanthanide ions were added to liquid chromatography systems in either a pre- or a post-column mode [9,10]. By this method, the selection of a mobile phase to obtain optimum elution should comply with the requirements of lanthanide luminescence. Paper strips with pre adsorbed europium(III) or terbium(III) were used for the simultaneous TRL determination of TC and ciprofloxacin [11] and for the determination of anesthetics, benzocaine and procaine [12]. Solid-phase microextraction was combined with room-temperature phosphorescence using Whatman 1PS phase separating paper [13] and solid-phase extraction was combined with time-resolved laser induced fluorimetry for screening polycyclic aromatic hydrocarbons [14]. Paper or cellulose membranes are most frequently used as the preferred media in solid-phase luminescence [12,13]. However, a sorbent which also can function as a spectroscopic substrate is still desired.

In this study four potential sorbents, C18, pectin, cellulose and filter paper, were examined for this purpose. TC, as a model analyte, was extracted from aqueous solutions by the sorbents and directly detected by SMTRL on the surface after coordination with europium(III) nitrate. The best candidate sorbent was selected and an analytical procedure was devised.

2. Materials and methods

2.1. Reagents and solutions

Thin-layer chromatography (TLC) plates, C18 and cellulose (all without fluorescent indicator) and all chemicals and biological reagents were purchased from Sigma-Aldrich (San Louis, MO). Tetracycline hydrochloride, europium(III) nitrate pentahydrate, disodium ethylenediaminetetraacetic acid dihydrate (Na_2EDTA) and tris(hydroxymethyl)aminomethane (Tris) were analytical reagent grade and used without further purification. Filter paper, Whatman 42, was obtained from Fisher Scientific (Pittsburgh, PA). Deionized water (D.I. water) was prepared using a Barnstead E-pure water system (Dubuque, IA) and used to prepare all aqueous solutions.

0.10 M Tris–2.5 mM EDTA buffer solution was prepared by dissolving 12.11 g of tris(hydroxymethyl)aminomethane in 900 ml D.I. water, the solution pH was adjusted to 9.0 using 5 N HCl, 25.0 ml 0.1 M Na_2EDTA was then added and the solution was diluted to 1.0 L with D.I. water. TC stock solution was prepared in acetonitrile at 100 ppm and stored at 4 °C. TC working solution at 1 ppm was prepared daily from the TC stock solution by dilution with either Tris–EDTA buffer or D.I. water in an amber screw-capped vial. In TC enrichment experiments, dilute TC in water solutions at the concentrations of 10 or 1 ng/ml were prepared directly in 500 ml Teflon beakers from the TC working solution. Eu(III) solution, 2.5 mM $Eu(NO_3)_3$–2.5 mM Na_2EDTA, was prepared either in 0.1 M Tris buffer at pH 9 or in D.I. water at pH 7.

2.2. Sorbent preparation

The C18 TLC plate used is a thin layer of silica gel derivative backed by a glass substrate. The plate was cut into 25 mm × 8 mm strips; the silica layer was carefully cut to a 10 mm × 6 mm rectangle in the central portion. Cellulose (backed with a polyester film) and filter paper were cut into 10 mm × 6 mm rectangles and mounted on glass strips (25 mm × 8 mm) using double-sided adhesive tape.

Pectin gels were cast from a pectin solution. Pectin (degree of esterification, 93%; content of galacturonic acid, 63%; from citrus peel) solution was prepared by suspending pectin powders in D.I. water at 2% (w/v), followed by stirring at room temperature for 18 h. The pectin solution, 100 μl, was evenly loaded on a glass strip (25 mm × 8 mm) and freeze-dried. This "loading-drying" cycle was repeated four times. The pectin layer was trimmed to 10 mm × 6 mm, rinsed with 0.5 M $CaCl_2$ and D.I. water, 10 ml for each, and air-dried. All solid surfaces were prepared in a clean environment and stored in a desiccator at room temperature.

2.3. Apparatus

A commercial fluorescence spectrometer (Model Cary Eclipse, Varian, Walnut Creek, CA) was used for SMTRL measurement. The instrumental operation and signal processing were handled by Cary Eclipse Lifetime software. The sample holder shown in Fig. 2 is modified from the original cuvette holder to accommodate the sorbent strip. The vertical mounting plate has a 10 mm × 6 mm (width × height) rectangular window opening to expose the surface of substrates to

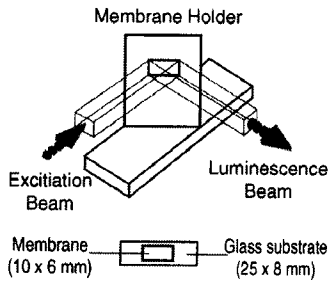

Fig. 2. Sample holder for solid-matrix time-resolved luminescence (SMTRL).

the excitation beam, which has a cross section of approximately 7 mm × 3 mm (width × height) in this position. Both the height of the window and the holder position were carefully adjusted to fully intercept the excitation beam and to maximize the signal beam intensity received by the photomultiplier tube in the detector compartment. The excitation beam incidence angle was optimized at 60°. To minimize stray light, the front surface of the mounting plate was painted flat black. The glass strip was mounted against the back of the holder plate using two binder clips, with the surface exposed to the light through the window.

2.4. Scanning electron microscopy (SEM)

All solid surfaces for TC extraction and SMTRL measurement were pre-frozen at −20 °C overnight and lyophilized at −78 °C for 24 h. Dry samples were mounted on aluminum specimen stubs with double-sided adhesive tape. The glass side was facing downward while the surface side was coated with a thin layer of gold by DC sputtering. Images of topographical features were made using a model JSM 840A scanning electron microscope (JEOL USA, Peabody, MA) coupled to an Imix-1 digital image workstation (Princeton Gamma Tech, Princeton, NJ). The average pore size of solid surfaces was measured directly on the SEM micrographs and calculated as described previously [15].

2.5. SMTRL for TC determination

The SMTRL measurement was performed with two different reagent-loading sequences. For one sequence, 10 μl of Eu(III) solution (pH 9) was first loaded onto the surface followed by drying over calcium sulfate in a dark desiccator at room temperature for 30 min; then spotted with 10 μl of TC working solution, and dried again. For another sequence, Eu(III) and TC were loaded in a reversed order. After sample loading, the sorbent strip was mounted on the sample holder. The SMTRL was measured at room temperature at the following setting: excitation wavelength: 388 nm, emission wavelength: 615 nm, both excitation and emission slit widths: 20 μm. For each run, ten cycles were collected and averaged. The signal intensity was integrated over 60–800 μs interval. The luminescence lifetime was calculated.

For comparison studies, the drying process was skipped in some experiments, SMTRL measurements were done on wet solid surfaces right after reagent loading.

2.6. TC adsorption

For dilute TC solutions, TC enrichment was accomplished by repeated spotting or immersion with TC solution. For repeated spotting, 10.0 μl of 1.0 ng/ml TC solution was loaded onto a Eu(III) pre-absorbed solid surface and dried in a desiccator. The spotting-drying cycle was repeated 10 times prior to SMTRL measured. For the immersion approach, three pieces of Eu(III) pre-loaded sorbent strips were placed into a Teflon beaker containing 450 ml TC solutions at 1.0 or 10.0 ng/ml. After gently shaking at 4 °C for desired time intervals, the solid sorbents were removed, rinsed with D.I. water, re-loaded with Eu(III) if necessary, and measured by SMTRL.

Alternatively, TC was desorbed from the solid surfaces by washing with methanol and the amount of desorbed TC was determined in aqueous solution by TRL [13]. Briefly, each sorbent strip was treated with 2.0 ml methanol solution (methanol:acetonitrite:D.I. water; 5/3/2, v/v) by lightly sonicating for 10 min. The solution was concentrated to 0.4 ml by placing in a desiccator connected with vacuum line, adjusted to pH 9 using Tris–EDTA, then measured by TRL. The amount of adsorbed TC (N_s) and the amount of TC remaining in liquid phase after extraction (N_l) were used for the calculation of the distribution constant K, according to the formula $K = N_s V_l / V_s N_l = C_s / C_l$ [16]. Where, V_s is the volume of solid surfaces as estimated from their dimensions; V_l is the volume of liquid phase; C_s and C_l are the TC concentrations calculated for solid phase and aqueous phase, respectively.

2.7. Eu(III) desorption

The Eu(III) desorption from solid surfaces was evaluated by immersing the sorbent strips in D.I. water for desired time. The amount of Eu(III) remaining on the sorbent surface was determined by SMTRL after TC loading.

3. Results

3.1. Physical and chemical properties of solid phase surfaces

The four types of sorbents were examined for their architecture and composition in the context of TC adsorption and SMTRL measurement. Among these materials, C18, cellulose and filter paper are well known media in chromatography and have been used for TC analysis.

The micrographs of the sorbent surfaces are shown in Fig. 3. All samples were porous and the pores were interconnected. C18 and cellulose had smoother surface morphology than filter paper and pectin. C18 and cellulose had similar pore size, which was smaller than those found in pectin and filter paper. In addition, C18 and cellulose were characterized by a narrow pore size distribution. Pectin had the largest average pore size among all samples. The pores in pectin and filter paper were extremely uneven (Fig. 3, Table 1). Moreover, pectin had limited swellability upon contact with aqueous solution; the swelling behavior of other sorbents was negligible.

The four sorbents also differ from each other in chemical composition. C18 is a silica gel impregnated with hydrocarbon compounds to form nonpolar, hydrophobic zones within the gel and to coat the silica gel particles. Both cellulose and filter paper are the derivatives of natural plant polysaccha-

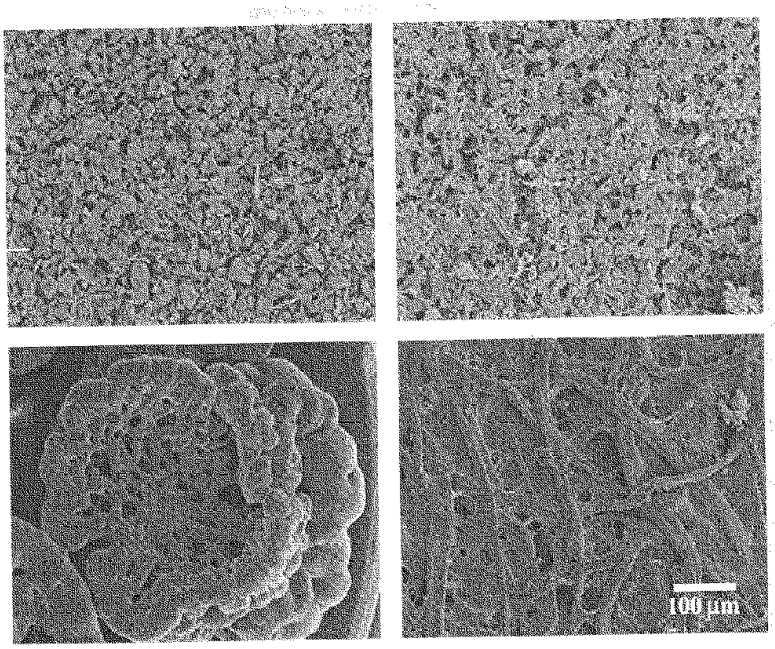

Fig. 3. SEM photographs of the four sorbents studied. Clockwise from top left: C18, cellulose, filter paper and pectin.

rides and are hydrophilic. The calcium cross-linked pectin is another polysaccharide-based sorbent consisting of both hydrophilic and hydrophobic domains. Hydroxyl groups are abundant on the surfaces of the three polysaccharide-based sorbents, while surface hydroxyl groups on the C18 are mostly blocked. The pectin also contains carboxylic groups, calcium, sodium and potassium ions.

3.2. SMTRL measurement

To investigate if a TC and Eu(III) complex can be formed on the sorbents, 10 μl of Eu(III) solution, pH 9, was first loaded onto the surface of a sorbent and SMTRL measured. Some background TRL signal was observed with minor differences in intensity among the sorbents. However, a strong TRL signal at 615 nm was recorded when 10 μl of 1.0 ng/μl TC solution was added to the surface following the Eu(III) loading. This phenomenon was observed for all tested sorbents. A typical example obtained on C18 is shown in Fig. 4. The results indicated the formation of TC/Eu(III) complexes on the sorbent surfaces and confirmed the intramolecular energy migration from excited state of TC donors to Eu(III).

To optimize the SMTRL measurement, luminescence of TC/Eu(III) complexes on C18 was measured under different conditions, including presence or absence of water, pH, reagent loading sequence, and Eu(III) concentration. As shown in Fig. 4, an obvious enhancement in TRL intensity was obtained from the dry solid surface in comparison with the wet one. In another experiment, we investigated the possibility to alter the binding site of TC to Eu(III) on solid surfaces using TC solutions of two different pH values. C18 surfaces were loaded with 10 μl of Eu(III) in D.I. water (pH 7.0), and the C18 were then divided into two groups. One was spotted with TC in Tris–EDTA buffer (pH 9) and the other with the same amount of TC in D.I. water. The SMTRL measurement showed a higher luminescence in the pH 9 group. However, the difference between the two groups was reduced by spotting an aliquot of Tris–EDTA buffer (TC-free, pH 9) to the second group (data not shown). Fig. 5 shows the luminescence signals received on C18 surfaces with different reagent loading sequences. A much higher intensity was obtained when Eu(III) solution was seeded onto the surface prior to TC loading.

Fig. 6 shows the effect of Eu(III) concentration on luminescence of TC/Eu(III). The luminescence increased with increasing concentration of Eu(III), reaching the highest intensity at 25 nmole of Eu(III). After that, increasing Eu(III) loading did not affect the luminescence. Although the mole ratio between the TC and Eu(III) in the complex is 1:1, preloaded Eu(III) in a large excess (as calculated, TC:Eu(III)

Table 1
Surface properties of the four sorbents

Matrix	Thickness (μm)	Volume of matrix (ml)	Pore size (μm)	Particle size	Smoothness
C-18	250	0.015	6	5–17	Smooth
Cellulose	100	0.006	6	2–20	Smooth
Filter paper	200	0.012	>2.7	>20	Rough
Pectin	300	0.018	>50	>20	Rough

Data obtained from providers, except those related to pectin, which was measured as described in the experimental section. Volumes of solid matrices were calculated from the dimensions.

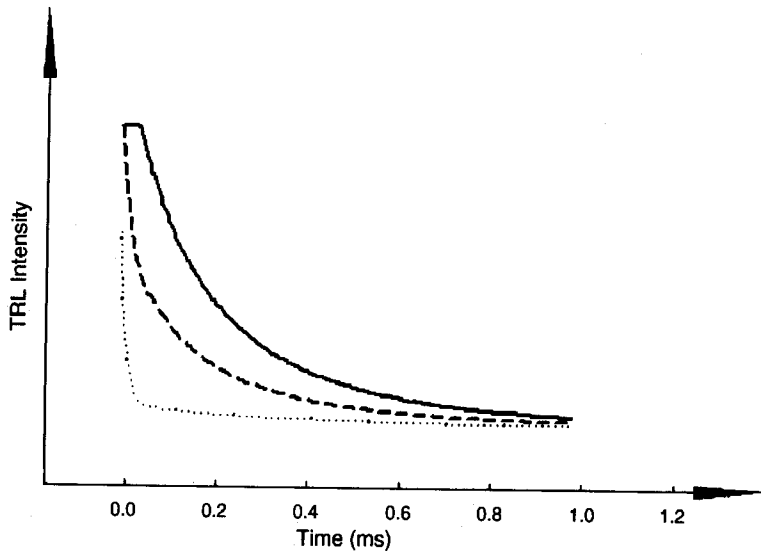

Fig. 4. Sensitivity of tetracycline detection by SMTRL method. Luminescence obtained in C18 from Eu(III) alone (dotted line) and TC/Eu(III) complex with (solid line) or without (dashed line) desiccation.

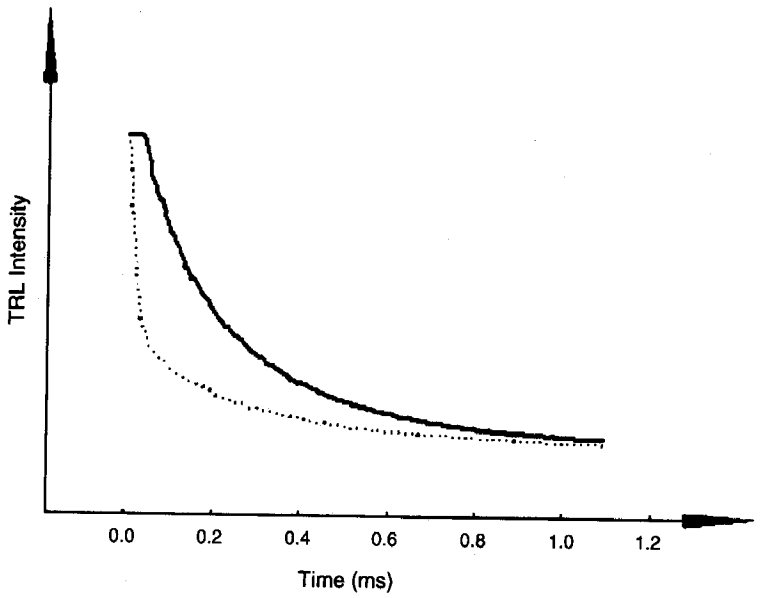

Fig. 5. Effect of reagent-loading sequence on SMTRL sensitivity detection. TC was spotted on Eu(III) pre-loaded C18 (solid line) and the opposite order (dotted line).

was 1:1, 250 when evaluated on a C18 surface) is essential to maximize SMTRL.

3.3. Comparison of sorbents for SMTRL and TC adsorption

As shown in Table 2, SMTRL signal intensity varied with the types of sorbents in the sequence of C18 > cellulose > pectin ≈ filter paper. The luminescence lifetime of TC/Eu(III) complex on the sorbent surfaces also varied in the same sequence.

Since Eu(III) pre-loaded solid surfaces demonstrated a higher SMTRL efficiency (Fig. 5), the capability of Eu(III)

Table 2
Luminescence of europium(III)–tetracycline adsorbed on various sorbents

Sorbents	Relative intensity	Lifetime (ms)
C-18	116.2 ± 1.4	>1.0
Cellulose	53.5 ± 10.9	>1.0
Filter paper	15.4 ± 2.1	0.8
Pectin	19.2 ± 2.9	0.7

25 nmol of Eu(III) in pH = 9 Tris buffer and 10 ng TC in D.I. water, were loaded into each matrix in sequence. The reagent loaded matrices were dried over drierite in a desiccator at room temperature for 30 min before SMTRL measurement. Data expressed as mean ± S.D. ($n = 3$).

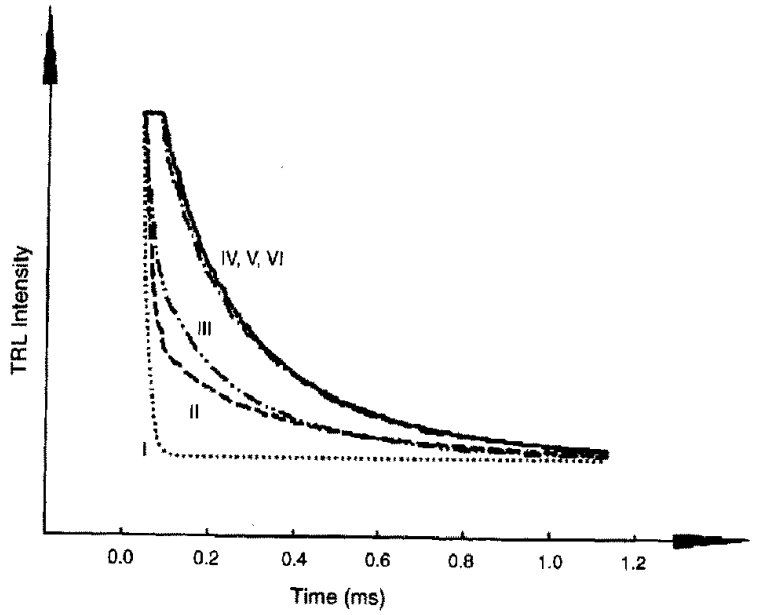

Fig. 6. Effect of Eu(III) content on TC/Eu(III) SMTRL measurement. The C18 was pre-loaded with Eu(III) of various amounts: 10 nmol (I, II), 20 nmol (I, III), 25 nmol (I, IV), 250 nmol (I, V) and 500 nmol (I, VI). 0.02 nmol TC was spotted to samples II–VI, and SMTRL measured.

pre-loaded sorbents in TC extraction was evaluated. First of all, the stability of pre-loaded Eu(III) in water had to be investigated. No desorption of Eu(III) from C18 was detected up to 12 h immersion in 150 ml D.I. water. In contrast, significant desorption of Eu(III) from other sorbents was detected after 12 h immersion (data not shown). These results demonstrated that the C18 retained Eu(III) stronger than the other three sorbents.

Fig. 7 shows the kinetics of TC extraction by C18 and cellulose. With Eu(III) pre-loaded C18, TC adsorption increased rapidly in the beginning, and reached a plateau in 12 h, indicating equilibrium was achieved. With cellulose, TC adsorption increased slowly. At equilibrium the amount of TC found on cellulose was much lower than that on C18. Table 3 shows the capability of all sorbents in TC extraction. By the immersion approach, TC was detectable on C18 at 1.0 ng/ml level, while detectable for all other solid surfaces at the 10 ng/ml level. At 10 ng/ml, the signal on C18 was about 12 times those on cellulose, filter paper or pectin. By the repeated spotting approach with 10×10 µl TC solution, TC was detectable on all sorbents even at 0.1 ng, the luminescence intensity increased in the same order of sorbents as

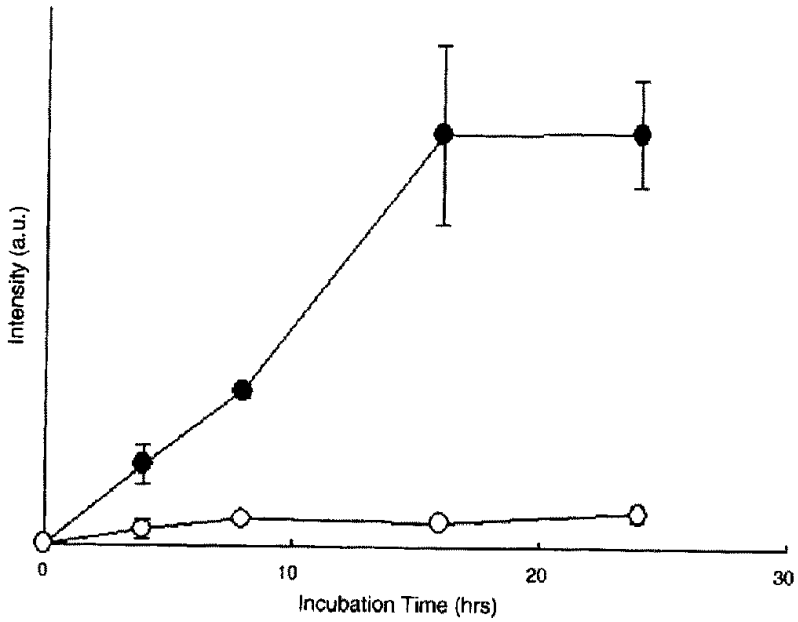

Fig. 7. Enrichment of TC from 10 ng/ml TC solution by C18 (●) and cellulose (○). Each sorbent (10 mm × 6 mm) was pre-loaded with Eu(III), immersed in 150 ml of TC solution and shaken at 4 °C for given period. At the end of experiments, the matrices were removed, rinsed with D.I. water, desiccated and analyzed by SMTRL. For cellulose, an additional 25 nmol of Eu(III) were spotted on the surface after its removal from water. All tests were in triplicate.

Table 3
Luminescence and sensitivity of TC enriched by two loading methods

Matrix	SMTRL intensity			LOD[a] (ng/ml)	Linear range (ng/ml)
	Immersion model (ng/ml)		Repeated spotting model, 10 ng		
	1	10			
C-18	9.3 ± 3.6	231 ± 30.2	17.5 ± 0.4	0.7	0–1000
Cellulose	N/D[b]	19.2 ± 4.2	8.3 ± 2.7	5.2	0–700
Filter paper	N/D	15.3 ± 3.6	2.7 ± 0.5	–	–
Pectin	N/D	17.7 ± 3.9	2.5 ± 0.7	–	–

[a] The limits of detection (LOD) were calculated by $LOD = 3S_B/m$, where S_B is the standard deviation of the blank measurements and m is the plot slope of SMTRL intensities versus concentration. Both LOD and linear range were obtained with the solid matrices on which TC was loaded by repeated spotting method.

[b] N/D: not detectable.

Table 4
Calculated distribution constants K of TC for the four sorbents

Matrix	C18	C18 with pre-loaded Eu(III)	Cellulose	Filter paper	Pectin
$K = C_s/C_l$	490	640	254	127	1270

C_s and C_l: the concentrations of TC on solid matrices and liquid phase, respectively. TC on the solid matrices was desorbed by methanol washing and the amount was determined by TRL measurement in aqueous solution.

shown by the immersion method. In comparison to cellulose, a lower LOD and a larger linear range were obtained for C18.

The TC distribution between aqueous phase and extraction phase is shown in Table 4. The distribution constant is a measure of affinity of the extraction phases to the analyte. A higher K value indicates a higher affinity between the sorbent and TC. Calcium cross-linked pectin was able to extract much more TC from the solution than other solid surfaces; C18 also had strong affinity for TC adsorption; both the cellulose and filter paper were very poor in TC adsorption.

4. Discussion

We have demonstrated that TC can be enriched by a sorbent and directly determined on the same sorbent by Eu(III)-sensitized TRL. Among the four sorbents tested, C18 showed beneficial properties in both sample preparation and analysis. The SMTRL measurement has several advantages over TRL conducted in solution. First of all, the SMTRL measurement only requires a small volume of sample and has high sensitivity (Table 2). Secondly, the use of solid phases minimizes the effect of water quenching on SMTRL (Fig. 4). Water molecules quench the luminescence in solution by occupying lanthanide coordination sites [17,18], therefore a synergistic co-ligand is required to diminish the effect of water quenching. With thin layer solid surfaces, adsorbed water can be removed simply by exposing the sorbents to dry air or a vacuum. The simple procedure could be attributed to the porous structure of the sorbents, which allows a quick liquid migration.

Another advantage of SMTRL is that the complex formation can be regulated directly on the sorbents. As mentioned in the results section, the addition of TC in D.I. water to Eu(III) pre-adsorbed C18 generated low fluorescent intensity recovable by spotting a small volume of higher pH solution on the solid surface after reagents loading. The pH dependence of SMTRL is reflected by the changes in the binding site of TC. TRL of TC and lanthanide complexes in aqueous phase is highly pH-dependent. At neutral or acidic pH, the preferential binding site in TC to Eu(III) is the enolic hydroxy group on the A ring (Fig. 1). In the solution with pH higher than 7.5, the hydroxy groups on the B, C, D rings of TC are deprotonated, thus responding to chelate bonding with the lanthanide ion (Fig. 1) [6,9,19]. The binding at B, C, D sites places the Eu(III) closer to the benzoyl moiety, resulting in an efficient energy migration and thus enhances luminescence intensity. Apparently, the binding position of TC to Eu(III) ions pre-adsorbed on the C18 can be switched from one to another by loading a small volume of aqueous solution with different pH, just as in liquid phase. It is expected that the architecture of C18 provides spaces in favor of the interaction and the liquid retained in pore areas of the solid phase facilitates the chelate bonding between TC and Eu(III).

The surface properties of solid phases, in terms of architecture and chemical composition, have an influence on TC enrichment and SMTRL. A nearly two-fold higher luminescence was observed for C18 than for cellulose with a single spot of 10 ng TC (Table 2) or 10 spots of 0.01 ng TC (Table 3). The spotting method ensures consistent reagent application to all surfaces, hence the changes in TRL intensity reflect the influence of solid surfaces on SMTRL. Both C18 and cellulose matrices are similar in physical characteristics, such as the similar pore size and particle size, and similar surface smoothness, but differ in chemical composition (Table 1, Fig. 3). It suggests that the chemical composition may play a key role in SMTRL. Likewise, pectin has the chemical structure and surface functional groups which are similar to filter paper and cellulose; the TRL intensities observed on the three surfaces resulted in the same relative order. The TRL intensities observed on filter paper and pectin were slightly lower than on cellulose; it could be attributed to the difference of their surface smoothness, as smooth surfaces tend to yield a higher signal-to-noise ratio [20].

To compare the amount of TC extracted by different sorbents with the immersion method, TC also was measured by

TRL in aqueous solution after desorption. This avoided the effect of surface-to-surface variation. The sorbent-dependent adsorption activity revealed by TRL measurements (Table 4) is different from that by SMTRL analysis (Table 3), indicating the effect of the surface properties of sorbents on TC/Eu(III) luminescence. For those sorbents with higher K value and lower SMTRL intensity, they may have a lower TC/Eu(III) population or a strong environmental quenching might occur [20,21]. Accordingly, the calcium cross-linked pectin is excellent in TC extraction, but not suitable for SMTRL application. Cellulose and filter paper are not the selection for both TC enrichment and SMTRL. Although the influence of solid surface on luminescence could be directly or indirectly due to surface functionalities, sugar components, impurities or physical properties, more detailed research is needed.

The interference of surface properties of a sorbent to TC/Eu(III) luminescence could be minimized by pre-loading Eu(III) ions in excess to the surface. As shown in Fig. 5, a much higher intensity was obtained on Eu(III) pre-loaded C18 than from plain C18. As mentioned above, at pH of about 9 the chelate bonding of TC occurs at positions near the benzoyl moiety, which also is responsible for TC binding to the hydrophobic C18. Supposedly, the adsorption of TC on C18 may reduce the accessibility of B, C, D rings to Eu(III) chelation. As a result, the efficiency of SMTRL is reduced. This was compensated by seeding a large amount of Eu(III) on the C18 surface. The TC molecules that were added after Eu(III) loading are most likely to be captured by the pre-adsorbed Eu(III) ions rather than landing on the bare C18 surface, resulting in a higher TC/Eu(III) complex population on the surfaces, and therefore a higher SMTRL signal. Further evidence for this mechanism was obtained by increasing the amount of pre-loaded Eu(III) on the sorbent surface (Fig. 7). The pre-loading of Eu(III) to C18 may have created a pseudo surface, which facilitated the chelate bonding of TC to Eu(III) and the luminescence.

5. Conclusions

We have found that TC could be measured on the surface of a C18 TLC plate by the SMTRL method. The pre-adsorption of Eu(III) on the C18 facilitated TC/Eu(III) complex formation and the subsequent luminescence. The C18 also showed a high affinity in TC adsorption. Thus, TC analysis could be simplified by the hyphenation of TC extraction and TC measurement using a single C18. This method can be applied for routine screening TC and other luminescent lanthanide-chelating analytes in environmental or biological conditions. In comparison with C18, cellulose and filter paper are poor in TC extraction and TC/Eu(III) luminescence; calcium cross-linked pectin facilitates TC adsorption but interferes with the SMTRL luminescence. The architecture and composition of C18 seem to be responsible for its distinct advantages in TC analysis. The definite surface properties such as chemical inactivity, relative hydrophobicity and smoothness, as well as an appropriate porosity are considered as typical features of the optimal solid phase, which can fulfill the dual purposes as a sorbent for analyte extraction and a substrate for analyte detection by SMTRL. A procedure to use C18 sorbent strip for TC determination in milk has been successfully developed based on the method described in this study [22].

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Title: High-Pressure Inactivation of Hepatitis A Virus Within Oysters

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High-Pressure Inactivation of Hepatitis A Virus within Oysters

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Previous results demonstrated that hepatitis A virus (HAV) could be inactivated by high hydrostatic pressure (HHP) (D. H. Kingsley, D. Hoover, E. Papafragkou, and G. P. Richards, J. Food Prot. 65:1605–1609, 2002); however, direct evaluation of HAV inactivation within contaminated oysters was not performed. In this study, we report confirmation that HAV within contaminated shellfish is inactivated by HHP. Shellfish were initially contaminated with HAV by using a flowthrough system. PFU reductions of >1, >2, and >3 $\log_{10}$ were observed for 1-min treatments at 350, 375, and 400 megapascals, respectively, within a temperature range of 8.7 to 10.3°C. Bioconcentration of nearly 6 $\log_{10}$ PFU of HAV per oyster was achieved under simulated natural conditions. These results suggest that HHP treatment of raw shellfish will be a viable strategy for the reduction of infectious HAV.

Bivalve shellfish readily bioconcentrate microbial pathogens from marine and estuarine waters. While fecal coliforms and other pathogenic bacteria from human and animal wastes do not persist within shellfish tissues beyond a few days, enteric viruses such as hepatitis A virus (HAV) can persist in estuarine waters and within shellfish tissues for periods of several weeks or more (1, 4, 15, 28, 38). In fact, HAV and other viruses are readily identified in shellfish harvested in certain European regions (3, 10, 17, 29, 31). HAV-contaminated shellfish have caused significant outbreaks of human disease (5, 11, 22). Furthermore, global trade of virally contaminated shellfish has resulted in outbreaks and dissemination of HAV and other exotic virus strains to geographic areas where they are not endemic (5, 19, 27, 35, 36).

Once virus contaminated, there are limited postharvest options for inactivating infectious virus within shellfish while retaining the raw characteristics and high market value of the product. A commercial process called depuration, in which live shellfish stock are placed in tanks of clean seawater for periods of up to several days, is generally recognized as inadequate for purging HAV and other enteric viruses (10, 18, 21, 28, 38). Irradiation is of limited utility, because inactivation of enteric viruses requires relatively high levels of radiation that can negatively affect shellfish taste, appearance, and shelf life (16, 23). Presently, cooking is generally recognized as the only reliable mitigation method to sanitize shellfish potentially contaminated with HAV (13, 32). However, many shellfish consumers insist on eating raw shellfish or at least minimally cooked oysters, because cooking alters the organoleptic qualities of shellfish.

Recently, an alternate technology, high hydrostatic pressure (HHP), has come to the forefront as a potential means for mitigating pathogens within raw shellfish. HHP is utilized commercially on the United States gulf and western coasts (Gold Seal Oysters Inc., Homa, La., and Nisbet Oyster Inc., Bay Center, Wash.) at pressures of up to 275 megapascals (MPa), principally because it can facilitate the oyster-shucking process and extend the shelf-life of raw oysters due to the reduction of spoilage bacteria (24). Organoleptic evaluations have shown that HHP-treated oysters are acceptable to consumers at treatment pressures as high as 400 MPa or approximately 60,000 lb/in^2 (30).

Experimental evidence suggests that HHP can inactivate some important bacterial pathogens. Berlin and coworkers (2) demonstrated that *Vibrio vulnificus*, *V. parahaemolyticus*, and *V. cholerae* were inactivated in artificial seawater by 15-min treatments at 250 MPa. In this same study, a 10-min, 200-MPa treatment of homogenized raw oysters inoculated with 10^7 CFU of either *V. vulnificus* or *V. parahaemolyticus*/g reduced the bacteria to levels of <10 CFU/g. Cook (12) has recently extended this work to demonstrate that naturally occurring *V. vulnificus* in both whole oysters (*Crassostrea virginica*) and oyster meat homogenates were reduced by ~5 log after pressure treatment at 241 MPa for 120 s.

With respect to viral pathogens, it was demonstrated that feline calicivirus, a norovirus surrogate, can be inactivated by HHP at pressures of 275 MPa (26) and that San Miguel sea lion virus 17 (SMSV-17), a second norovirus surrogate, was inactivated by HHP. In oyster homogenate, the titer of SMSV-17 was reduced by 0.04, 1.57, 3.35, and >3.97 $\log_{10}$ PFU/ml at 200, 250, 275, and 300 MPa, respectively, when pressurized for 1 min (7).

For HAV, previous work using 5-min treatments has shown limited inactivation at 300 MPa in cell culture media. Treatments of 460 MPa resulted in a 7 $\log_{10}$ reduction of HAV to nondetectable levels (26). In this study, we investigated the potential of HHP to inactivate HAV directly within shucked

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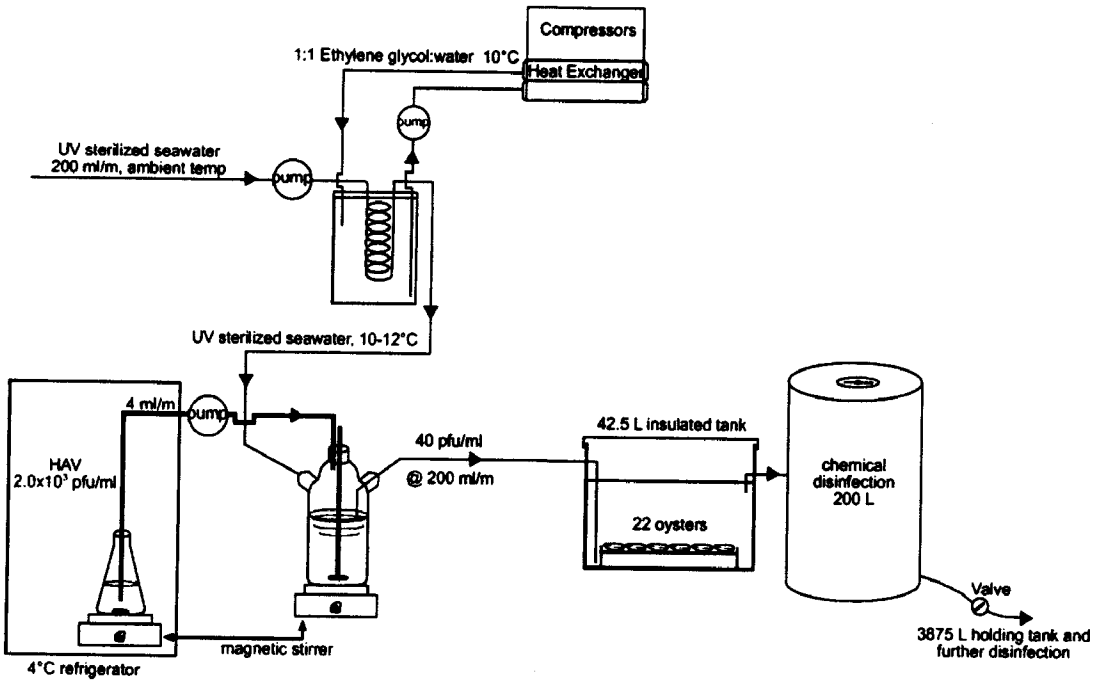

FIG. 1. Diagram of a seawater flowthrough unit designed for contamination of oysters with HAV. Oysters were exposed to HAV in groups of 22 oysters per trial. Three oysters were selected for each pressure treatment and assay.

oyster meats after contamination with HAV in a flowthrough natural seawater system.

(A preliminary report of this work was presented at the 90th Annual International Association for Food Protection meeting in New Orleans, La., August 2003.)

MATERIALS AND METHODS

HAV and oysters. HAV virus strain, HM-175, was propagated in fetal rhesus monkey kidney (FRhK-4) cells (14). Working stocks were propagated on confluent FRhK-4 cells in Eagle's minimum essential medium (MEM) supplemented with 15% fetal bovine serum, 15 mM HEPES, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.11% sodium bicarbonate, 0.1 mg of kanamycin/ml, and 0.05 mg of gentamicin/ml (Invitrogen Corp., Carlsbad, Calif.). Cells were infected (multiplicity of infection [MOI] = 1) in medium containing 2% fetal calf serum, and after propagation for 2 weeks, lysates were obtained after three freeze-thaw cycles. This was followed by an extraction with equal volumes of chloroform. One-milliliter portions supplemented with 2% fetal bovine serum were frozen at −80°C.

Eastern oysters (*C. virginica*) were harvested from an approved area in Mobile Bay, Ala. After culling and sorting, 200 commercial-size oysters were placed into a depuration flume at the U.S. Food and Drug Administration (FDA) Gulf Coast Seafood Laboratory, Dauphin Island, Ala. Oysters were maintained for more than 3 weeks prior to being transferred to a flume which utilized single-pass UV-treated natural seawater. Salinities ranged from 5 to 20 ppt.

Three days before virus accumulation, 22 oysters were placed in the accumulation tank to acclimatize to 10°C. This tank received UV-treated seawater which had been refrigerated to 10°C at the rate of 200 ml/min in laminar flow (Fig. 1). Temperature and flow conditions that optimize natural virus accumulation were previously determined by Burkhardt and Calci (6). HAV (7.1 $\log_{10}$ PFU) was added to 6 liters of sterile RO (reverse osmosis) water and was continually mixed at 4°C. Peristaltic pumps (Cole-Parmer, Vernon Hills, Ill.) combined 4 ml of virus suspension/min with 200 ml of UV-treated seawater/min in a cytostir vessel (Kimble, Vineland, N.J.). The calculated overall concentration within the accumulation tank was 40 PFU/ml. After 24 h, the oysters were divided into six groups of three oysters each and were shucked into sterile cups. The total weight for each group was approximately 25 g.

High-pressure treatment. Shucked oyster samples were transferred into 4.5-ml Scotchpak pouches (Kapak 500, Minneapolis, Minn.) and heat sealed using an Impulse Food Sealer (American International Electric Co., Whittier, Calif.) according to the manufacturer's instructions. An overpack 2-mm pouch was sealed over the inner pouch. Refrigerated, shucked oyster samples were packed in accordance with International Air Transport Association Dangerous Goods Shipping Regulations in an biohazard shipping container (STP 100; SAF-T-PAK, Alberta, Canada) and enclosed in an insulated carton with blue-ice packs to insure that the temperature remained at <10°C during shipping. This was verified by including a continuous recording digital thermometer (RD-temperature; Omega, Stamford, Conn.) in several shipments. Shipments were by overnight carrier to the U.S. FDA, National Center for Food Safety and Technology, Summit Argo, Ill., for processing. Pressurization of oyster samples was carried out for 1 min using a Quintas Model QFP-6 high-pressure food processor (ABB Autoclave Systems, Inc., Columbus, Ohio). Samples were pressurized at 300, 325, 350, 375, and 400 MPa for 1 min at approximately 9°C using a 50/50 mix of water and ethylene glycol medium. The come-up times to reach final pressures and temperature parameters for each sample group are shown in Table 1. Pressure release time was almost immediate (<3 s). After processing, the refrigerated samples were shipped overnight to the USDA Microbial Food Safety Research Unit at Dover, Del., for virus extraction and assay.

Virus extraction and plaque assays. Virus-contaminated shellfish (three shellfish per group) were removed from pressurized sealed pouches, placed in 50-ml conical tubes, and briefly centrifuged in a table top centrifuge to facilitate separation of oyster meat from oyster liquor. Uncontaminated (negative) and nonpressurized (0 MPa) HAV-contaminated (positive) controls were also tested.

TABLE 1. Physical parameters during HHP treatment

Pressure (MPa)	Trials (*n*)	Mean temp, °C (SE)			CUT[a] (SE)
		Initial	Maximum	Final	
300	3	9.9 (0.3)	17.7 (0.6)	17.6 (0.5)	79 (4.5)
325	3	9.3 (0.1)	17.8 (0.3)	17.7 (0.4)	80 (1.2)
350	3	9.6 (0.7)	18.5 (0.8)	18.5 (0.8)	83 (0.9)
375	3	9.1 (0.3)	18.8 (0.5)	18.8 (0.5)	89 (1.5)
400	3	9.0 (0.3)	19.6 (0.9)	19.5 (0.9)	94 (2.7)

[a] CUT, Come-up time or length of time in seconds needed to reach set pressure.

TABLE 2. Viable HAV detected after HHP treatment

Pressure (MPa)	Average $\log_{10}$ PFU (SE)	$\log_{10}$ PFU (SE) for trial no.:		
		1	2	3
0	5.82 (0.48)	5.71 (0.07)	5.29 (0.03)	6.45 (0.01)
300	5.58 (0.26)	5.42 (0.19)	5.37 (0.10)	5.95 (0.02)
325	5.04 (0.19)	4.78 (0.09)	5.16 (0.08)	5.20 (0.07)
350	4.54 (0.06)	4.62 (0.06)	4.49 (0.13)	4.51 (0.03)
375	3.50 (0.14)	3.63 (0.14)	3.3 (0.26)	3.56 (0.25)
400	2.67[a] (1.12)	2.82 (0.45)	<1.5	3.69 (0.12)

[a] HAV was not detected in one trial, so the detection limit 1.5 $\log_{10}$ was assumed.

Virus extractions were performed as described by Kingsley and Richards (28). Two milliliters of extract or 2 ml of 10-fold serial dilutions were made in Earle's balanced salt solution, and plaque assays were performed in triplicate using FRhK-4 cells as described by Richards and Watson (33).

RT-PCR. The glycine, polyethylene glycol, trireagent, poly(dT) magnetic bead (GPTT) method for viral RNA extraction was adapted from Kingsley and Richards (25). Essentially, 0.15 M Na_2HPO_4 (pH 9.5) was substituted for glycine buffer for extraction of oyster meats, with the remainder of the procedure being the same. For reverse transcription-PCR (RT-PCR) amplification of HAV sequences, primers originally described by Robertson et al. (34), (+) *2949* 5'TATTTGTCTGTCACAGAACAATCAG3' and (B) *3192* 5'AGGAGGTG GAAGCACTTCATTTGA5', were used. Conditions for RT-PCR and the use of heat-denaturated HAV virions as positive RT-PCR controls were the same as those previously described (25). The 267-bp major amplification product was observed for HAV-contaminated samples in all groups after treatment. All uncontaminated oyster samples tested negative by RT-PCR for HAV.

Analysis of data. Data from three 1-min trials with HAV-contaminated shellfish (Table 2) were plotted as groups at 300, 325, 350, 375, and 400 MPa as a function of the logarithmic reduction in HAV titer compared to that of untreated samples (Fig. 2). Trend lines were determined using the curve-fitting graphing program Sigma Plot, version 8.02, for Windows (SPSS Inc., Chicago, Ill.). Analysis of slope covariance was performed using the SAS program (SAS Institute, Cary, N.C.). HAV titers were determined in triplicate. The average PFU obtained was determined, with standard error expressed logarithmically (Table 2).

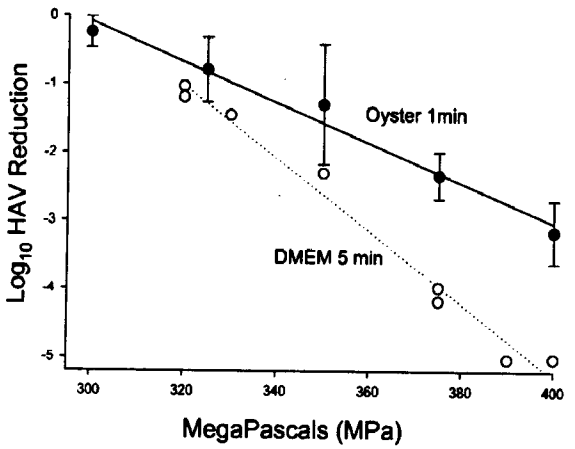

FIG. 2. Effect of pressure treatments on HAV within contaminated oysters. HAV-contaminated oysters were administered 1-min pressure treatments at pressures ranging from 300 to 400 MPa (dark circles, solid line). Average extractable HAV reductions are displayed graphically as a logarithmic reduction from three trials. HAV error bars represent standard errors for titer reductions from three trials. Reductions within oyster tissues are graphically compared with previously published results (26) for 5-min treatments of HAV in DMEM with 10% fetal bovine serum (dashed line, open circles). The two slopes displayed were determined to be significantly different ($P < 0.05$).

RESULTS

Live oysters were contaminated with HAV to $>10^5$ PFU/oyster in three separate experimental trials. During each of three trials, HHP, ranging from 300 to 400 MPa in 25-MPa increments, was administered to five treatment groups of three oysters each for 1 min. In this study, maximal and final adiabatic temperature increases during pressure treatments were recorded and are shown in Table 1. Results indicate that there was an average of 10.6°C adiabatic heating effect at 400 MPa with maximal temperature not exceeding 20°C. As HAV is highly thermostable, readily resisting temperatures of >60°C, these results confirm that inactivation observed for HAV is not due to adiabatic heating during pressurization.

Virus was extracted from all groups by using phosphate buffer. Extracted HAV was assayed by plaque assay. Results for all three trials are shown in Table 2. Levels of HAV achieved within oyster meats, as measured by HAV extraction from three non-pressure-treated oyster samples per trial, averaged 5.82 $\log_{10}$ extractable PFU. The average titer obtained at each pressure is displayed in Table 2 (three oysters/group) and as a $\log_{10}$ reduction plot in Fig. 2. The detection limit for the cell culture assay was approximately 33 PFU/oyster group or 1.5 $\log_{10}$, as 6 ml of the original 200 ml of extract was assayed for viable virus. This value was assumed for virus extractions which yielded no detectable virus when constructing Table 2 and Fig. 2.

Viral RNA extraction via the GPTT procedure was performed on all shellfish extracts. All samples exposed to HAV tested positive by RT-PCR. All noncontaminated oysters tested negative for HAV by RT-PCR, indicating that oysters were originally free of HAV. It was previously demonstrated that pressure-treated HAV maintains the integrity of its RNA (26).

DISCUSSION

Although previous results demonstrated the potential effectiveness of HHP against HAV in cell culture media (26), it was necessary to directly confirm that HAV can be inactivated within the context of a contaminated oyster, because food matrix composition, such as fat content, water activity, and salt concentration, can affect HHP pathogen inactivation rates (9, 26, 37). Conceivably, HAV could be found in a number of different anatomical structures within the shellfish, such as being bound to shellfish mucus membranes, within the digestive gland contents, encased within phagocytic cells, or free floating within the open circulatory system of the bivalve. Results reported here with oysters contaminated with $>10^5$ PFU demonstrate that the virus is inactivated by HHP within these environments, as judged by a 3 $\log_{10}$ reduction of virus at 400 MPa for 1 min. Results from experiments using oysters contaminated with $\sim 10^3$ to 10^4 PFU (data not shown) yielded no infectious virus after 400-MPa treatment, indicating that lesser concentrations of HAV can be completely inactivated within shellfish.

HHP inactivation results for HAV within oysters are similar to results obtained previously with 5-min treatments with a high-titer HAV stock in DMEM with 10% fetal bovine serum (26). The previous inactivation curve obtained is plotted as a

dashed line in Fig. 2. In both experiments, a linear inactivation curve was obtained when the $\log_{10}$ of virus titer was plotted with pressure treatments above 300 MPa. For the HAV-contaminated oysters, the inactivation rate was not as great as that obtained previously with HAV in DMEM. Beyond the obvious oyster matrix and salinity differences, there may be a number of potential explanations for this. First, pressure treatments performed in the present study were for 1 min rather than 5 min. Although the pressure levels are the predominating factor determining the degree of inactivation of virus, it has been shown that increased treatment time at a given pressure will enhance the amount of virus inactivation observed (9, 26). Second, in this study oysters were pressurized at an initial temperature of 9°C for 1 min. In contrast to previous experiments with HAV (26), pressurization of samples was performed in an oil-based pressure unit at room temperature (21 to 22°C). The influence of temperature on HHP effectiveness against viruses has not been defined in this study.

For oysters, it is conceivable that physiological or environmental factors, such as water salinity, may alter HHP effectiveness. Shellfish, unlike marine vertebrates, do not osmoregulate, hence their intracellular ionic strength mimics that of the estuarine or marine environment. *C. virginica* oysters are indigenous to areas with salinities ranging from 5 to 30 ppt, and salt content can vary considerably depending on the harvest area or can even vary within the same harvest area due to fluctuations in rainfall. Oysters tested here were from Mobile Bay, Ala., a low-salinity estuary, and they were maintained in approximately 5- to 20-ppt-salinity seawater.

Unlike experiments performed here, commercial high-pressure processes use whole shellfish (within shell) rather than shucked product. It was not possible to use whole HAV-contaminated oysters due to the potential of the sharp shell edges to puncture the containment bags. No appreciable differences of inactivation between shucked meats and whole-shell oysters are envisioned, because HHP is uniformly applied and the bivalve's shells cannot form a protective airtight seal.

Although the molluscan bivalve's ability to concentrate viruses and bacteriophages from contaminated water is well documented, use of the flowthrough unit with natural estuarine water confirms that oysters can and do concentrate HAV to quite high levels (above 5 $\log_{10}$) in a relatively short period of time (24 h). In fact, it has been suggested that virus bioconcentration rates from contaminated water to shellfish tissues can be as high as 1,000-fold on a per-gram basis (8, 20). Actual levels of HAV and other viruses achieved during natural contamination events would be a function of virus concentration within the contaminated water, duration of exposure, shellfish pumping, and metabolic rates, as well as virus inactivation rates within oyster tissues and the water column.

The quantity of virions, or PFU, that constitute an infectious dose or the amount of HAV in shellfish typically associated with outbreaks have not been determined. It would seem probable that shellfish legally harvested from approved growing areas would not be grossly contaminated. Presumably, a logarithmic reduction in PFU after HHP treatment would result in a concomitant reduction in infectious dose. Therefore, high-pressure treatment capable of reducing infectious doses 1,000-fold (3 $\log_{10}$) would probably be sufficient to render all but the most highly contaminated shellfish safe for consumption without cooking. Human fecal viruses do not replicate within shellfish tissues, therefore virus levels cannot increase as a result of temperature abuse after harvest or HHP treatment.

While we view application of HHP technology to raw shellfish as an important potential means of inactivating HAV and perhaps other pathogens, we do not envision HHP as a direct alternative to proper shellfish harvest water classification under the U.S. National Shellfish Sanitation Program or the European Union shellfish fecal coliform meat standard. Rather, we suggest that HHP, applied in addition to present sanitation standards, could provide an added measure of safety to shellfish designated for raw consumption and/or cooking.

In summary, this study demonstrates that HAV can be inactivated within the environmental context of the oyster and suggests that this technology has strong potential as an intervention strategy for shellfish sporadically contaminated with HAV. Additional studies evaluating HHP effectiveness of several types of shellfish from different water salinities, physiological states, and seasons may be necessary. Lastly, a means of directly demonstrating HHP effectiveness against norovirus would be especially beneficial.

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Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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Title: Enzyme-Linked Immunomagnetic Electrochemical Detection of Live *Escherichia coli* O157:H7 in Apple Juice

Author(s): A. G. Gehring, and S.I. Tu

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Research Note

Enzyme-Linked Immunomagnetic Electrochemical Detection of Live *Escherichia coli* O157:H7 in Apple Juice†

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ABSTRACT

We describe the application of enzyme-linked immunomagnetic electrochemistry (ELIME) for the rapid detection of *Escherichia coli* O157:H7 in buffered apple juice. The ELIME technique entails sandwiching bacterial analyte between antibody-coated magnetic beads and an alkaline phosphatase–conjugated antibody. The beads (with or without bound bacteria) were localized onto the surface of magnetized graphite ink electrodes in a multiwell plate format. The enzyme substrate, 1-naphthyl phosphate, was added, and conversion of substrate to an electroactive product was measured using electrochemical detection. With this technique, detection of whole, live *E. coli* O157:H7 bacterial cells was achieved with a minimum detectable level of ca. 5×10^3 cells per ml in Tris-buffered saline or buffered apple juice in an assay time of ca. 80 min. With adjustment of pH, the ELIME response for the bacteria in either sampling medium was similar, indicating that apple juice components did not contribute to any discernible sample matrix effects.

Rapid bacterial detection methods have been developed as alternatives to lengthy and laborious, yet selective and highly sensitive conventional culture techniques *(7, 8, 11)*. Some of these methods have employed the selectivity of immunoassays and both the rapidity and sensitivity of electrochemistry for the detection of molecular analytes or bacteria *(4, 5, 10, 12)*. In addition, several methods have combined the selectivity and potentially high surface area–mediated capture capability of immunomagnetic beads (IMB) with electrochemistry for the detection of molecular analytes or bacteria *(9, 13, 16, 17)*.

In this study, we have applied enzyme-linked immunomagnetic electrochemistry (ELIME) to the rapid detection of whole *Escherichia coli* O157:H7 cells in either buffer or apple juice. Apple juice has been selected as a target sample matrix due to the past association of the pathogenic bacteria, *E. coli* O157:H7, with outbreaks of food poisoning and hemolytic uremic syndrome linked to the commodity *(1–3)*. The ELIME methodology involved sandwiching of *E. coli* O157:H7 cells between IMB and alkaline phosphatase–conjugated antibody. The IMB (with or without bound bacteria) were localized onto graphite ink strip electrodes with the aid of permanent magnets. Enzyme substrate (1-naphthyl phosphate) then was added, and conversion to an electroactive product was quantified using Osteryoung square wave voltammetry.

MATERIALS AND METHODS

Materials. Materials used in this research included alkaline phosphatase–conjugated goat anti–*E. coli* O157:H7 (anti–*E. coli* O157:H7 conjugate, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), *E. coli* O157:H7 B1409 (Centers for Disease Control and Prevention, Atlanta, Ga.), goat anti–*E. coli* O157 M-280 IMB, magnetic particle concentrator (Dynal Inc., Lake Success, N.Y.), graphite ink silk screen printed on Mylar (1-cm wide graphite ink strips separated by 3 mm on Mylar sheets measuring 31 by 38 cm, The Motson Co., Inc., Flourtown, Pa.), Alnico magnets (cylindrical 4.8-mm diameter by 25.4 mm, Edmund Scientific Co., Barrington, N.J.), 1-naphthyl phosphate (1-NP; disodium salt, 98%) and platinum wire (0.25-mm diameter, 99.99%, Aldrich, Milwaukee, Wis.), 1% Blocker casein (Pierce, Rockford, Ill.), brain heart infusion (Difco, Becton Dickinson, Sparks, Md.), and double coated plastic Tuck Carpet Installation Tape (Tesa Tape Inc., Charlotte, N.C.). Other chemicals used were of reagent grade. Pasteurized apple juice ("100% juice reconstituted from concentrate") that contained no added preservatives or sweeteners was purchased from a local vendor.

Apparatus. All reactions with shaking were performed on a Vortex-Genie 2 (Scientific Industries, Bohemia, N.Y.). Bacteria samples were counted on a Petroff-Hausser bacteria counting chamber (Thomas Scientific, Swedesboro, N.J.). Electrochemistry of samples was performed in a custom-built multiwell electrode/magnet assembly that was constructed of polymethyl methacrylate blocks, Alnico magnets, double-sided tape, and graphite ink strip electrodes as previously described *(9)*. All electrochemical measurements were obtained with a BAS CV-50W electrochemical analyzer (Bioanalytical Systems, Inc., West Lafayette, Ind.) with accompanying BAS 50W software (version 2.0). An Ag/AgCl reference electrode (0.6 cm by approximately 7 cm, Vycor tipped, Bioanalytical Systems, Inc.), wrapped with a platinum wire that

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served as a counter electrode, was inserted into the tested solutions during electrochemical measurements.

Growth and enumeration of *E. coli* O157:H7. A loopful of *E. coli* O157:H7 cells collected from a slant was inoculated into 25 ml of brain heart infusion broth and incubated at 37°C for 18 h. A portion of the cells was serially diluted in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, pH 7.6) to 1:10 and 1:100 dilutions. Bacteria in an aliquot (6 μl) of the 1:100 dilution were enumerated using only the center grid (0.2 by 0.2 mm) of a Petroff-Hausser counting chamber. Enumeration was repeated three times using additional 6 μl aliquots of the 1:100 dilution, and a mean (± standard deviation) was determined for the four obtained values. The 1:100 dilution was used for further serial dilution in the preparation of samples for the generation of calibration curves. The subsequent error (standard deviation) in the determined bacteria concentration (mean value for the 1:100 dilution) was propagated taking into account an estimated error of 5% for volumetric measurements.

Purification of 1-NP. To reduce electrochemical background response associated with oxidation of 1-napthol possibly present in 1-NP, the 1-NP was purified using the following procedure: 50 mg of 1-NP (gray crystals) was placed into a glass tube and dissolved in 0.5 ml of methanol (high-performance liquid chromatography grade); 50 mg of charcoal was added along with an additional 0.5 ml of methanol; the mixture was warmed to approximately 37°C for 10 min followed by gravity filtration through filter paper (qualitative 2; Whatman Limited, England) into a glass vial; 2 ml of warmed methanol was used to rinse the test tube and filter paper; nitrogen gas was passed over the filtrate until the solvent was evaporated, and the walls of the vial were scraped with a metal spatula to retain the white 1-NP powder that was stored at −20°C until used.

ELIME detection of bacteria. *E. coli* O157:H7 bacteria contained in a 1:100 dilution (in TBS) of stock were further serially diluted in either TBS or unbuffered apple juice. Where indicated, the pH of the apple juice samples was then adjusted to 7.6 using 1 M Tris (76 μl per ml of apple juice). With reaction volumes suggested by Dynal Inc., 20 μl of IMB were placed in 1.5-ml polypropylene microcentrifuge tubes, 1 ml of bacteria (in either TBS or apple juice) was added, and the mixture was incubated by shaking (minimal agitation sufficient to prevent settling of the IMB) for 30 min. The tubes were placed into the magnetic particle concentrator for 3 min in order to trap the IMB (a portion containing bound bacteria when present) against the walls of the tubes, and the liquid was removed by aspiration. (During IMB concentration, the particle concentrator was gently inverted several times to retain any IMB located in the cap of the tubes and to focus the IMB into a single spot.) The IMB were resuspended by gentle vortexing with 1 ml of anti–*E. coli* O157:H7 (diluted 1:500 in 1% Blocker casein) for 30 min. The IMB again were separated using the magnetic particle concentrator for 3 min, and the liquid was removed. The IMB then were washed and resuspended twice with 1 ml of TBS followed by magnetic separation for 3 min in the magnetic particle concentrator and removal of the liquid after each wash. Finally, the IMB were resuspended with 0.2 ml of TBS.

For electrochemical analysis, 200 μl of IMB (with or without bound bacteria) were added to the solution holding block of a multiwell electrode/magnet. The beads were magnetically trapped against the electrodes for 2 min, and the liquid was removed by aspiration. With the magnetic field applied, 200 μl of 1-NP (2.7 mM in 0.2 M Tris, pH 9.6) was added to the well and allowed to react for 3 min. Production of electroactive 1-napthol was measured using Osteryoung square wave voltammetry (50 to 500 mV, 25 mV sweep width amplitude, 5 Hz frequency, 4 mV step potential, 10^{-5} A/V sensitivity) and the peak current was determined by drawing a tangent line across the base of the peak using the BAS 50W software. The electrochemical detection of immunomagnetically captured bacteria is schematically represented in Figure 1.

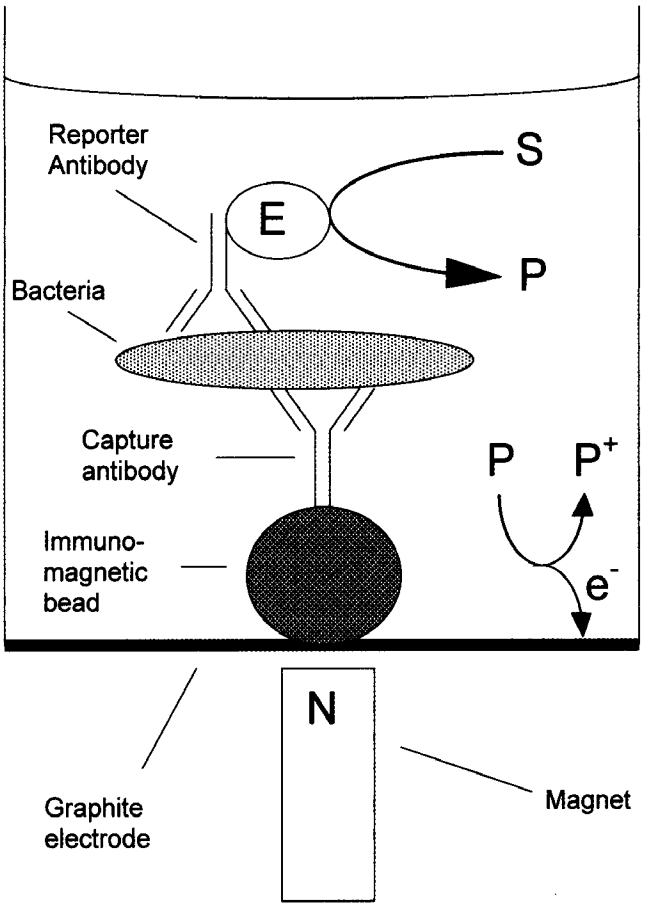

FIGURE 1. *Schematic representation of the enzyme-linked immunomagnetic electrochemical (ELIME) assay. Multi-ply immunogenic analyte (bacteria, for example) is sandwiched between antibody-coated magnetic beads (IMB) and enzyme-labeled antibody conjugate (reporter antibody). IMB (with or without bound bacteria) are magnetically trapped onto the electrode surface and exposed to substrate, and electroactive product is electrochemically detected. E, enzyme; S, substrate; P, product; and P^+, oxidized product.*

RESULTS AND DISCUSSION

The ELIME procedure entails the capture of multiantigenic analyte (e.g., bacteria) by IMB, labeling with enzyme (e.g., alkaline phosphatase)–conjugated antibody in a sandwich immunoassay format, magnetic concentration of the sandwiched bacteria at an electrode, and subsequent electrochemical analysis after a brief reaction with an electroactive enzyme substrate (Fig. 1). In a preliminary experiment, ELIME was used for the detection of live *E. coli* O157:H7 inoculated into untreated apple juice as compared to buffer (TBS). The results, portrayed as the mean (± standard deviation) electrochemical response (current) of triplicate measurements for one experiment, are displayed

TABLE 1. *Enzyme-linked immunomagnetic electrochemical (ELIME) detection of live* E. coli *O157:H7 (bacteria) in pristine, Tris-buffered saline (TBS) versus unbuffered apple juice*[a]

Sample	No. of bacteria	ELIME response (current; μA)	Standard deviation (current; μA)
TBS buffer blank	0	0.05819	0.02340
TBS buffer	$<4 \times 10^5$/ml	10.36	3.040
Apple juice blank	0	0.04308	0.02480
Apple juice	$\sim 4 \times 10^5$/ml	3.812	1.120

[a] Bacteria ($\sim 4 \times 10^5$/ml) were inoculated into TBS or untreated apple juice and analyzed using ELIME. The table values represent average responses of triplicate measurements ± standard deviation.

in Table 1. The presence of weak acids (ascorbic, citric, malic) in the apple juice accounted for an initial pH of approximately 3.6 for the juice. The dramatically reduced (approximately 60% lower) ELIME response observed for bacteria inoculated into untreated apple juice relative to TBS was presumed to be due to relatively poor recovery of the bacteria by the IMB, hence, pH inhibition of immunological reaction.

Results dictated that the pH of apple juice samples should be adjusted to that of the TBS (pH 7.6) prior to reaction with IMB. Based upon the titration of apple juice with standardized NaOH, at least 58 meq of base would have to be added to neutralize the weak acids contained therein, and a relatively small, additional amount of base would be required to further raise the pH to 7.6. Tris base solely was employed to maintain the same base system as used in the comparative TBS and since the resultant ionic strength would be similarly sufficient in prevention of potential bacterial cell lysis by osmotic pressure. Interpolation of the titration results (data not shown) indicated that 76 μl of 1 M Tris was required to adjust 1 ml of apple juice to pH 7.6.

Further experiments were performed to compare ELIME calibration curves for live *E. coli* O157:H7 in buffer (TBS) versus buffered apple juice. The experimental results, representing the ELIME detection of whole, live *E. coli* O157:H7 cells in TBS or Tris-buffered apple juice is displayed in Figure 2. Figure 2 displays the electrochemical response versus the number of *E. coli* O157:H7 bacteria per milliliter tested. ELIME detection of the bacteria exhibited very similar responses (sensitivity and slopes) for either of the sample matrices. The error displayed for the electrochemical response (current) was the standard deviation from the mean for measurements in triplicate for one experiment, whereas the error in the bacteria concentrations was derived from combining the error associated with enumeration of the initially diluted bacteria solution with estimated volumetric errors of 5% propagated over the range of serial dilutions.

The total assay time, based on a single sample, was approximately 80 min for ELIME. However, since the samples are concurrently reacted in a multiwell format, only approximately 5 min is required for the analysis of additional samples. Conservatively estimating the limit of detection to be 10 times the standard deviation of the blank response added to the blank response, substitution in the respective regression lines yielded limits of detection for *E. coli* O157:H7 to have been 3,300 cells per ml (in TBS) and 1,500 cells per ml (in buffered apple juice). The performance of ELIME for the detection of *E. coli* O157:H7 in apple juice favorably compares with other reported methods: 1,000 to 2,000 cells per ml in <1 h *(17)* and approximately 10^3 to 10^4 cells per ml in <1 h *(15)*. However, a risk assessment study *(6)* of the prevalence of the pathogen in apple cider indicates very low levels of potential contamination (i.e., 3 to 9 CFU/1,000 apples). Hence, as has been shown in past reports *(14, 18)*, enrichment culture must be employed prior to detection with select biosensors

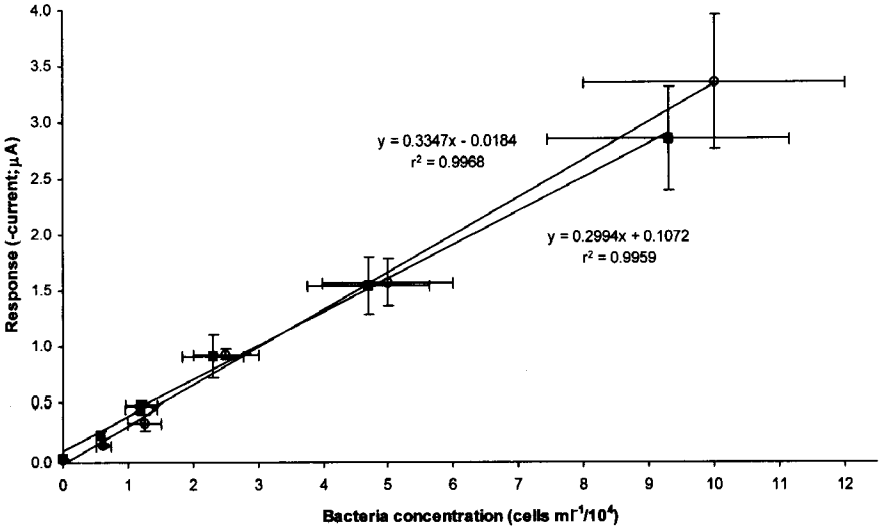

FIGURE 2. *ELIME detection of whole, live* E. coli *O157:H7 cells (bacteria).* E. coli *O157:H7 cells were serially diluted in either TBS or apple juice (pH adjusted with 1 M Tris as described in "Materials and Methods"), aliquots (1 ml) were immunomagnetically captured with anti–*E. coli *O157:H7 IMB, reacted with alkaline phosphatase–labeled anti–*E. coli *O157:H7 antibody conjugate, and detected electrochemically as previously described. The plot displays the electrochemical (current) responses for the varying concentrations of bacteria (○, in TBS; ■, in pH adjusted apple juice) that were tested.*

and biosensor-based methods (including ELIME in its current state of development) in order to achieve superior detection limits of ca. 2 to 10 CFU/ml or less.

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Title: Bacterial Poly(hydroxyalkanoate) Polymer Production from the Biodiesel Co-Product Stream

Author(s): R.D. Ashby, D.K.Y. Solaiman, and T.A. Foglia

Citation: Journal of Polymers and the Environment (2004) 12:(3) 105-112

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Bacterial Poly(hydroxyalkanoate) Polymer Production from the Biodiesel Co-product Stream

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A co-product stream from soy-based biodiesel production (CSBP) containing glycerol, fatty acid soaps, and residual fatty acid methyl esters (FAME) was utilized as a fermentation feedstock for the bacterial synthesis of poly(3-hydroxybutyrate) (PHB) and medium-chain-length poly(hydroxyalkanoate) (*mcl*-PHA) polymers. *Pseudomonas oleovorans* NRRL B-14682 and *P. corrugata* 388 grew and synthesized PHB and *mcl*-PHA, respectively, when cultivated in up to 5% (w/v) CSBP. In shake flask culture, *P. oleovorans* grew to 1.3 ± 0.1 g/L (PHA cellular productivity = 13–27% of the bacterial cell dry weight; CDW) regardless of the initial CSBP concentration, whereas *P. corrugata* reached maximum cell yields of 2.1 g/L at 1% CSBP, which tapered off to 1.7 g/L as the CSBP media concentration was increased to 5% (maximum PHA cellular productivity = 42% of the CDW at 3% CSBP). While *P. oleovorans* synthesized PHB from CSBP, *P. corrugata* produced *mcl*-PHA consisting primarily of 3-hydroxyoctanoic acid ($C_{8:0}$; 39 ± 2 mol%), 3-hydroxydecanoic acid ($C_{10:0}$; 26 ± 2 mol%) and 3-hydroxytetradecadienoic acid ($C_{14:2}$; 15 ± 1 mol%). The molar mass (M_n) of the PHB polymer decreased by 53% as the initial CSBP culture concentration was increased from 1% to 5% (w/v). In contrast, the M_n of the *mcl*-PHA polymer produced by *P. corrugata* remained constant over the range of CSBP concentrations used.

KEY WORDS: Poly(hydroxyalkanoates); Poly(3-hydroxybutyrate); *Pseudomonas oleovorans*; *Pseudomonas corrugata*; biodiesel.

INTRODUCTION

The production of animal fats (including lard, poultry fat, grease and edible and inedible tallow) and vegetable oils in the US has increased steadily over the past 10 years. In fact, today the Americas produce approximately one-third of the world's animal fats and two-thirds of the world's vegetable oils. This increase in animal fat and vegetable oil production has necessitated the discovery of additional outlets for these materials especially in non-food applications. One such outlet that is steadily gaining momentum around the world is the production of biodiesel (typically methyl or ethyl esters from animal fats and/or vegetable oils, Fig. 1). Between the years 1998 and 2002, world production of biodiesel increased from 0.2 to 32 million gallons and because of its fuel properties and improved emission characteristics, production is projected to reach 350 million gallons by the year 2011 [1]. Such increases in biodiesel demand, while providing an additional outlet for fats and oils, will result in a large co-product stream (Co-product Stream from Biodiesel Production, CSBP) that is composed

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Note: Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

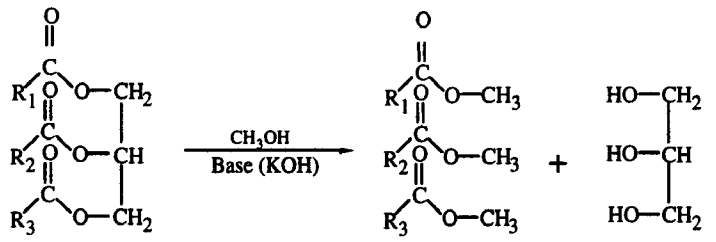

Fig. 1. The synthetic scheme (alkaline catalyzed transesterification) of biodiesel from animal fats and/or vegetable oils (R_1, R_2 and R_3 correspond to unique fatty acids attached to the glycerol backbone of the fat, oil or methyl ester).

primarily of glycerol, free fatty acids (FFA) and fatty acid methyl esters (FAME), the composition of which depends on the fat or oil feedstock, the transesterification process, and recovery efficiency of alkyl ester production.

Poly(hydroxyalkanoates) (PHAs) represent a complex class of naturally occurring bacterial polyesters that are synthesized intracellularly as carbon and energy reserve materials. Numerous bacterial species have been studied for their ability to synthesize PHA under the appropriate growth conditions. The most studied production system belongs to *Ralstonia eutropha,* which synthesizes short-chain-length PHA (*scl*-PHA) polymers (i.e., poly-3-hydroxybutyrate, PHB; or PHB-co-3-hydroxyvalerate, PHB/V) that behave as semicrystalline thermoplastics [2–4]. In contrast, many species of *Pseudomonas* belonging to the rRNA homology group I accumulate PHA polymers that are composed of either saturated or unsaturated 3-hydroxy fatty acid monomer units ranging in length from C6 to C14. These polymers, the so-called medium-chain-length (*mcl*-) PHA polymers, generally exhibit properties that range from elastomeric to adhesive-like depending on the specific side-chain length and the degree of unsaturation [5–10].

Previously, work has shown that different *mcl*-PHA-producing bacterial strains (primarily from the genus *Pseudomonas*) can utilize triacylglycerols [11–14] (*Aeromonas caviae* and *A. hydrophila* have been reported to synthesize a copolymer of PHB-co-3-hydroxyhexanoate from olive oil [15] and soybean oil [16], respectively), FFA (either pure [6–9, 17–19] or saponified oils [11, 20, 21]), or simple saccharides [19, 22–24]. Because the CSBP contains high concentrations of glycerol (a good bacterial growth substrate), FFA and FAME it seemed like a good material to support PHA synthesis under the appropriate growth conditions. In this study, we utilized two wildtype bacterial strains (*Pseudomonas oleovorans* NRRL B-14682 and *P. corrugata* 388) to convert CSBP into PHB or *mcl*-PHA, respectively, thus demonstrating a potential use for the CSBP in the development of additional value-added products.

MATERIALS AND METHODS

Materials

All simple salts were obtained from Sigma Chemical Company (St. Louis, MO). The CSBP was obtained from Ocean Air Environmental (Lakeland, FL) and was synthesized from the alkaline-catalyzed transesterification of soybean oil. All organic solvents used were HPLC grade and purchased from Burdick and Jackson (Muskegon, MI). The silylation reagent, N,O-bis(trimethylsilyl)-trifluoroacetimide (BSTFA), was purchased from Aldrich Chemical Company (Milwaukee, WI).

Strain Information and PHA Synthesis

P. oleovorans NRRL B-14682 was obtained from the culture collection of the NCAUR, ARS, US Department of Agriculture, Peoria, IL, while *P. corrugata* 388 was supplied by Dr. William Fett of the Eastern Regional Research Center, ARS, USDA (unless otherwise specified the strain designates will be omitted from this point on.) Stock cultures of each bacterium were prepared as described previously [22]. All shake flask experiments were conducted in 1-l Erlenmeyer flasks containing 500 mL volumes of Medium E* (pH 7.0) (for medium composition see ref. 8). CSBP (pH 13) was added to the sterile Medium E* in each test flask at concentrations between 1% and 5% (w/v) and the

pH's were readjusted to 7.0 ± 0.1 with predetermined volumes of 1 N hydrochloric acid. Seed cultures were prepared by inoculating 50 mL of Luria-Bertani (LB) broth with 0.5 mL of either *P. oleovorans* or *P. corrugata* from a thawed stock culture and incubating the cultures at 30°C, 250 rpm for 24 h. Polymer production experiments were carried out by inoculating each test flask with 3.0 ml of bacteria from the appropriate seed culture and incubating each flask at 30°C with shaking at 250 rpm for 72 hr. The cells were harvested by centrifugation (8000 × *g*, 20 min, 4°C), washed twice with deionized water and lyophilized (~24 h) to a constant weight.

CSBP Feedstock Composition and Utilization

CSBP feedstock was assayed for glycerol, fatty acid soaps (as FFA), and residual FAME. Glycerol (in mg/dl) was determined using the Glycerol-SL assay (Diagnostic Chemicals Limited, Charlottetown, P.E.I., Canada) according to manufacturer's specifications. FFA and FAME content were determined gravimetrically as follows: the alkaline CSBP (100 g) was acidified to pH 1 with concentrated hydrochloric acid and extracted with three 50 mL volumes of hexane. The hexane layers were combined into a tared flask, evaporated under nitrogen, and dried *in vacuo* (25 torr) for 24 h to constant weight. The flask was then reweighed and the amount of hexane solubles (FFA and FAME) was calculated by difference from the starting weight. The total concentration of FFA and FAME was determined by dissolving a known amount of hexane solubles back into hexane and using an established high performance liquid chromatography (HPLC) procedure [25].

To monitor selective utilization of the CSBP components by each bacterial strain during growth, the culture supernatants (after centrifugation) were assayed for glycerol, FFA, and FAME utilization. Glycerol was assayed as described above. FFA/FAME utilization was determined in triplicate on 10 mL aqueous samples taken directly from the culture supernatants. Each 10 mL sample was extracted with three 10 mL portions of hexane and dried as described previously. The control values for each component were derived from uninoculated test flasks that were incubated identically to the inoculated flasks. Glycerol and FFA/FAME consumption was calculated by difference from the values obtained from the uninoculated test flasks.

PHA Isolation

Intracellular PHA polymers were isolated from lyophilized cells by chloroform extraction at 30°C overnight with shaking at 250 rpm. Cellular debris was removed by filtration through Whatman #1 filter paper and the chloroform evaporated from the filtrate to give the crude polymer. The crude polymer was dissolved in a small volume of chloroform and reprecipitated by dropwise addition to cold methanol. The polymer was recovered, placed into a tared vial and dried *in vacuo* (25 torr) for 24 h.

FFA/FAME Compositional Analysis

The FFA and FAME composition of the hexane-soluble fraction of CSBP was determined by gas chromatography/mass spectrometry (GC/MS). Samples were redissolved in hexane at a concentration of 10 mg/mL and silylated by reacting 10 µL of each sample with 250 µL BSTFA and 200 µL of pyridine. The mixtures were heated at 70°C for 30 min and allowed to cool to room temperature. Finally, an additional 150 µL of hexane was added to each sample and the samples analyzed by GC/MS. Samples were injected (1 µL) into a Hewlett Packard (HP, Wilmington, DE) GC model 5890 Series II Plus equipped with a capillary inlet and an HP Mass Selective Detector (MSD) model 5972 Series set to scan from *m/z* 40 to *m/z* 550 at a rate of 1.5 scans/s. The capillary column (30 m × 0.25 mm) was coated with 0.25 µm of 5% cross-linked phenyl methyl silicone (HP-5MS). The oven temperature was programmed from 80°C (1 min) to 230°C (10 min) at 10°C/min. The injector port temperature was 250°C in the splitless mode and the detector transfer line was 280°C.

PHA Polymer Analysis

PHA repeat unit compositions were determined by GC/MS of the silylated 3-hydroxymethyl esters prepared from each PHA sample. Samples were prepared according to Brandl *et al.* [8] and were silylated and analyzed using the same conditions described above. Percent composition was obtained by reconstructing the chromatograms selecting the 175 ion, indicative of silylated 3-hydroxymethyl esters, and identifying the molecular ion -15 (CH_3 group; M-15) ions as described by Lee and Choi [26].

Molar mass averages were determined by gel permeation chromatography (GPC). The PHA

concentrations were 2.0 mg/mL for the *mcl*-PHA samples and 0.5 mg/mL for the PHB polymers. A Styragel HMW 6E (Waters Corp., Milford, MA) column was used with a calibration curve derived from polystyrene standards (Polyscience, Warrington, PA) with narrow polydispersities. Chloroform was used as the eluent at a flow rate of 1 mL/min and the injection volume in all cases was 200 μL.

RESULTS AND DISCUSSION

CSBP is primarily composed of glycerol, FFA and FAME. To quantitate the preferential utilization of these materials by *P. oleovorans* and *P. corrugata*, the concentration of each component in the starting material was determined and their consumption monitored as the cultures terminated. The CSBP used in this study was composed of 40% glycerol, 34% hexane-solubles (made-up of 92% FFA/ FAME and 6% mono- and diacylglycerols) and 26% water. Analysis of the hexane-soluble fraction by GC/MS indicated the presence of substantially higher amounts of FFA compared to FAME. Specifically, the hexane-soluble fraction was composed of the following ratios of FFA and FAME (in mol %): myristic acid, 1; palmitic acid, 18; stearic acid, 13; oleic acid, 25; linoleic acid, 25; and the methyl esters of palmitic acid, 2; stearic acid, 1; oleic acid, 7; and linoleic acid, 7. The higher concentrations of FFA compared to FAME (on a mol % basis) in the CSBP indicated that while the recovery rate of the biodiesel from the CSBP may have been less than optimal, the incomplete conversion of the starting oil to methyl esters probably caused the large hexane-soluble fraction in the CSBP. Based on the ratio of oleic acid and linoleic acid in the CSBP, we verified that the material was indeed derived from a soybean oil-based production system.

The fact that CSBP is a co-product that contains a large amount of glycerol (an easily useable substrate for bacterial growth) and FFA/FAME (widely used substrates for PHA production) made it a logical choice as a renewable, inexpensive substrate for PHA production. In the past, researchers have used the raw glycerol phase from biodiesel production containing 20% methanol as a substrate for PHB production [27]. In those studies, *Methylomonas extorquens*, a facultative methylotrophic bacterium, was used in a batch process to produce PHB from the glycerol phase of biodiesel production. It was determined that the organism initially metabolized methanol and then used glycerol for growth and PHB production. Unfortunately, the strain grew very slowly and resulted in low polymer yields. In this present study CSBP was tested as a sole carbon source up to 5% in simple salts media. Each culture was incubated for 72 h after which the cellular growth and PHA polymer yield were measured. It was found that both *P. oleovorans* and *P. corrugata* grew and produced PHA polymers from CSBP at media concentrations up to 5% (Fig. 2). Interestingly, increasing concentrations of CSBP had no effect on the cell growth of *P. oleovorans* ($CDW = 1.3 \pm 0.1$ g/L) but did cause a 100% increase in polymer yield (from 0.2 g/L at 1% CSBP to 0.4 g/L at 5% CSBP). On the other hand, the cell growth of *P. corrugata* decreased as the CSBP concentration increased. Specifically, *P.corrugata* showed maximum cell growth of 2.1 g/L at 1% CSBP, which decreased slightly to 1.7 g/L as the initial CSBP media concentration was increased to 5%. Additionally, polymer yields from *P. corrugata* stabilized at 0.7 g/L between 2% and 5% CSBP. Cell growth and polymer yield results indicated that the PHA cellular productivity trends for both *P. oleovorans* and *P. corrugata* were distinctly different (Fig 3). For example, the PHA cellular productivity for *P. oleovorans* increased at an average rate of 23% per 1% increase in CSBP concentration to a maximum PHA cellular productivity of 27% at 5% CSBP whereas, PHA cellular productivity of *P. corrugata* attained a maximum of 42% at 3% CSBP and remained constant up to 5% CSBP.

Previous work showed that *P. oleovorans* B-14682 and *P. corrugata* 388 contain the genetic makeup necessary to synthesize *scl*- and *mcl*-PHA, respectively [14,17]. In this study we rationalized that any PHA polymers synthesized by these strains would mimic the PHA produced by these organisms in past studies. To understand the mechanics behind the synthesis of PHA polymers by *P. oleovorans* and *P. corrugata*, we monitored the selective utilization of the glycerol, FFA, and FAME that made up the CSBP (Table I). The results showed that *P. oleovorans* preferred glycerol to FFA and FAME for growth and polymer production. In fact, *P. oleovorans* assimilated 1.7 g more glycerol on average than FFA or FAME. This was interesting in that glycerol is a more energetically favorable substrate for the formation of acetyl CoA (the precursor for PHB synthesis) than are FFA. This can be seen in the stoichiometry of the conversion of glycerol to acetyl CoA:

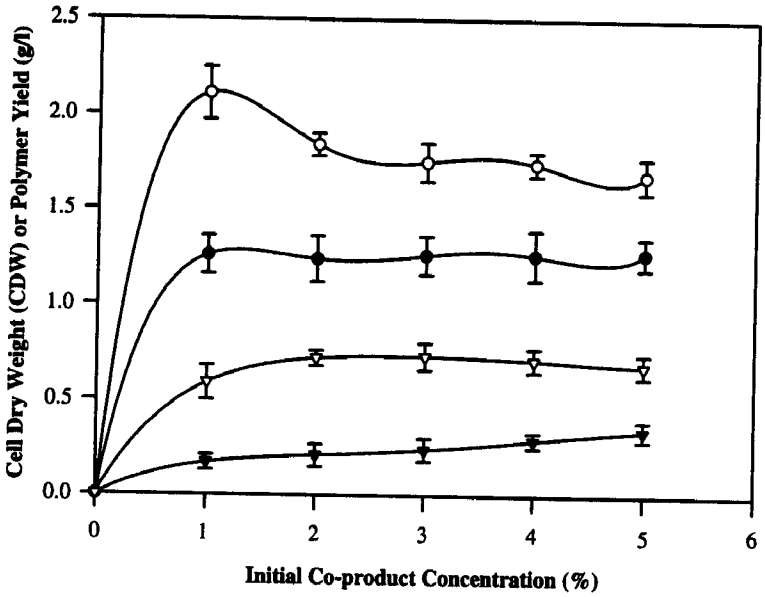

Fig. 2. The bacterial cell growth of *P. oleovorans* B-14682 (●) and *P. corrugata* 388 (○), the PHA polymer yield of *P. oleovorans* B-14682 (▼) and *P. corrugata* 388 (▽) grown in Medium E* on CSBP at concentrations from 1% to 5% for 72 h (n = 3).

$$\text{Glycerol} + 3\text{NAD}^+ + \text{ADP} + \text{CoA} \rightarrow \text{Acetyl CoA} + 3\text{NADH} + 3\text{H}^+ + \text{ATP} + \text{CO}_2 + \text{H}_2\text{O}.$$

The oxidative phosphorylation of NADH yields 3 ATP molecules per NADH molecule (total 9 ATP), resulting in a total net gain of 10 ATP molecules per acetyl CoA molecule synthesized from glycerol. In contrast, the energetics of acetyl CoA formation from 1 round of β-oxidation shows the net generation of only 4 ATP molecules per acetyl CoA formed. In short, 1 ATP molecule is required to activate the FFA

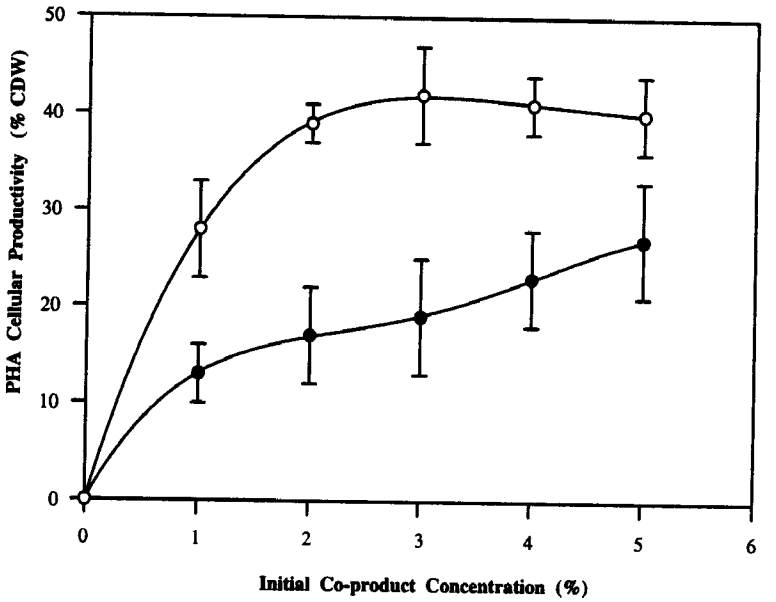

Fig. 3. The PHA cellular productivity [(polymer yield/CDW) × 100] of *P. oleovorans* B-14682 (●) and *P. corrugata* 388 (○) grown in Medium E* on CSBP at concentrations from 1% to 5% for 72 h (n = 3).

Table I. Preferential Carbon Source Utilization by *P. oleovorans* B-14682 and *P. corrugata* 388 on CSBP

	Substrate available (g)		Substrate utilized (g)		Substrate utilized (%)	
CSBP conc. (%)	Glycerol[a]	FFA/FAME fraction	Glycerol	FFA/FAME fraction	Glycerol	FFA/FAME fraction
P. oleovorans						
1	2.3	1.5	2.2	0.6	96	40
2	4.3	3.2	2.1	0.5	49	16
3	6.3	5.0	2.5	0.5	40	10
4	8.2	6.7	1.9	0.3	23	4
5	10.2	8.4	1.8	0.0	17	0
P. corrugata						
1	2.3	1.5	1.8	1.3	78	87
2	4.3	3.2	1.9	1.6	44	50
3	6.3	5.0	1.2	1.4	19	28
4	8.2	6.7	1.2	0.9	15	13
5	10.2	8.4	0.9	0.9	9	11

[a]Glycerol content was determined by the Glycerol-SL assay (Diagnostic Chemicals Limited, Charlottetown, P. E. I., Canada).
[b]FFA/FAME content was determined gravimetrically after extraction in hexane and drying under vacuum to constant weight (~24 h).

prior to the initiation of β-oxidation. However, once activated, 1 molecule of $FADH_2$ (which can be converted to 2 molecules of ATP upon oxidative phosphorylation) and 1 molecule of NADH are generated which results in a net increase of 4 ATP molecules per round of β-oxidation. The preference of glycerol utilization over FFA and FAME by *P. oleovorans* was further proven by GC/MS analysis of the FFA and FAME fraction of the remaining carbon source from the bacterial supernatant (Table II). It was found that the composition of the FFA/FAME fraction remaining after bacterial growth closely approximated the original material analyzed prior to inoculation, which further supported the preferential use of glycerol over FFA and FAME by *P. oleovorans*.

Carbon source utilization patterns for *P. corrugata* were slightly different from those of *P. oleovorans*. In fact, *P. corrugata* utilized both glycerol and FFA/FAME at approximately the same rate (Table I). Previous studies showed that *P. corrugata* contains the genes that catalyze the synthesis of *mcl*-PHA polymers. In the present case, each component of the CSBP could be used for separate applications by the organism. For instance, glycerol could be used as a growth substrate while the FFA and FAME could be β-oxidized to form the precursors for *mcl*-PHA biosynthesis. Analysis of the culture supernatant showed that the FFA and FAME composition was much different from that in the starting material (Table II). The most glaring difference was the decreased amount of oleic and linoleic acid and the increased concentration of methyl oleate and methyl linoleate. This suggested that the organism was methylating the free oleic and linoleic acids in the media. However, the increased variability between the starting FFA and FAME content and the FFA and FAME content after bacterial growth supports the increased utilization of FFA/FAME by *P. corrugata* and would further support the notion that the organism was primarily using the FFA/FAME as precursors for polymer formation.

Table II. FFA and FAME Composition of the Hexane-Soluble Fraction of the CSBP[a] after Growth of *P. oleovorans* B-14682 and *P. corrugata* 388 for 72 hr ($n = 3$)

	FFA (mol %)					FAME (mol %)			
Sample	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Uninoculated[b]	1±0	18±1	13±1	25±1	25±2	2±0	1±0	7±2	7±2
P. oleovorans	1±0	23±3	16±4	24±2	14±4	2±0	2±0	9±2	9±3
P. corrugata	1±0	19±2	16±2	19±3	2±0	4±0	4±1	15±3	20±3

[a]All measurements were made on the cultures containing 1% CSBP after harvesting the bacteria.
[b]The uninoculated flasks (controls) were incubated and assayed exactly as the test flasks.

Table III. Repeat Unit Composition of the PHA Polymers Synthesized by *P. oleovorans* B-14682 and *P. corrugata* 388 on CSBP (n = 3)

	3-Hydroxymethyl esters (mol%)									
CSBP conc. (%)	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{12:1}$	$C_{14:0}$	$C_{14:1}$	$C_{14:2}$	$C_{14:3}$
P. oleovorans										
1–5% CSBP	100	–	–	–	–	–	–	–	–	–
P. corrugata										
1–5% CSBP	1±0	4±0	39±2	26±2	4±1	3±1	1±0	5±1	15±1	3±0

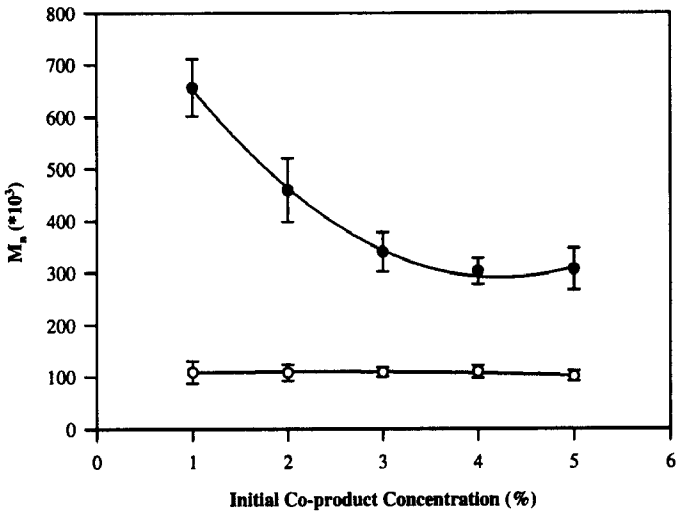

Fig. 4. The effect of initial CSBP media concentration on the number average (M_n) molar mass of the PHB derived from *P. oleovorans* B-14682 (●) and the *mcl*-PHA derived from *P. corrugata* (○) (n = 3).

Polymer analysis indicated that *P. oleovorans* did indeed synthesize PHB from CSBP whereas *P. corrugata* synthesized *mcl*-PHA (Table III). The composition of the *mcl*-PHA remained constant between 1% and 5% CSBP and was composed primarily of 3-hydroxyoctanoic acid ($C_{8:0}$, 39 mol%), 3-hydroxydecanoic acid ($C_{10:0}$, 26mol%) and 3-hydroxytetradecadienoic acid ($C_{14:2}$, 15 mol%). Medium-chain-length PHA generally reflects the composition of the substrate (FFA chain length and unsaturation) from which it was synthesized. The high concentration of $C_{14:2}$ was further indication that the CSBP used in this study was obtained from a soybean oil-based process. Interestingly, the molar masses of the polymers also exhibited different trends (Fig. 4). The *mcl*-PHA derived from *P. corrugata* had a number average (M_n) molar mass of 107,000 g/mol, which remained constant regardless of the CSBP concentration. In contrast, the M_n of the PHB from *P. oleovorans* grown on 1% CSBP was 656,000, but this value decreased by 53% as the CSBP concentration increased to 5%. There have been a number of examples of PHA molar mass control either through the use of media additives [28–30] or genetic manipulation of the producing strain [31], but the reason for the molar mass decrease in the PHB synthesized by *P. oleovorans* in this study is unknown at this time. Chemically induced chain termination has not been ruled out, and is the subject of further study.

In conclusion, we have shown that the CSBP can be used as a carbon substrate to synthesize both PHB and *mcl*-PHA depending on the bacterial strain used. To date, production yields have not been optimized, but this work establishes that CSBP can be utilized as a substrate for PHA polymer production without the need for separating and recovering the glycerol, FFA, and FAME. This innovative use of this valuable co-product stream further supports the conversion of fats and oils to biodiesel.

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Title: Application of Transglutaminase to Derivatize Proteins:1. Studies on Soluble Proteins and Preliminary Results on Wool

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Application of transglutaminase to derivatize proteins: 1. Studies on soluble proteins and preliminary results on wool†‡

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Abstract: The use of enzymes in chemical processing is gaining favour due to the reduction of hazardous chemicals and because it is considered to be environmentally safe. The acyl transfer reaction between primary amines and glutamine residues in proteins is catalysed by the enzyme transglutaminase. The efficiency of microbial transglutaminase to attach functional amines and catalyse inter- and intra-molecular crosslinks was investigated using reduced carboxymethylated κ-casein, gelatin and wool. Model systems used in this research gave evidence of both cross-linking of the protein and covalent binding of the primary amine *o*-phosphorylethanolamine to the protein. These data agree with earlier publications that show transglutaminase catalyses the formation of covalent cross-links between the γ-carboxyamide group of glutamine and the ε-amino group of lysine and also the incorporation of primary amines into proteins. Preliminary analysis of treated wool indicated the covalent bonding of the functional amine to the protein. Our goal is to increase the value of wool by enzymatic addition of functional groups to the wool fibre.

Keywords: *o*-phosphorylethanolamine; transglutaminase; gelatin Blooms

INTRODUCTION

The use of enzymes in the processing of agricultural commodities has steadily increased. Enzymes are used in numerous ways to improve functional properties of foods and fibres.[1] The use of enzymes in the processing of agricultural products is viewed as an environmentally safe procedure when the same results are achieved without exposure to harsh chemicals. Enzymatic approaches have been used to improve the properties of wool, gelatin and collagen hydrolysate as inexpensive and environmentally safe methods.[2,3] The field of industrial biocatalysis was recently reviewed by Zaks.[4] The use of enzymes in the textile industry is well known and accepted; the applications and technologies are many and varied.[5,6]

In the textile industry, enzymes are used in the biofinishing of cotton/wool blends.[3] Protein–glutamine, amine γ-glutamyl-transferase (EC 2.3.2.13), commonly known as transglutaminase (TGase), is an enzyme that catalyses the acyl transfer reaction in which a γ-carboxyamide group of a peptide-bound glutamine (Gln) residue is an acyl donor. The acyl receptor is a primary amine group, usually the ε-amino group of a lysine (Lys) residue;[7–9] *in vivo* this results in the modification of proteins through either intra- or inter-molecular crosslinking. *In vitro* the enzyme also catalyses the covalent attachment of primary amines to Gln residues,[10,11] as illustrated in Fig 1. Mammalian TGases are calcium-dependent.[12] As a class, TGases from various sources display varying reactivity towards Gln residues.[12] Microbial TGase, which was used in this study, differs from the mammalian enzyme in that it is calcium-independent, has a smaller molecular weight and is commercially available.[13] Claims that TGase may improve the quality of wool as in felting, whitening, handling and shrinking have been made.[14] It has been reported that the ability to incorporate various functional groups into the glutamine residues of protein using TGase would be very useful[15] as it might improve and enhance the end use of the protein. In this study we report the incorporation of a primary

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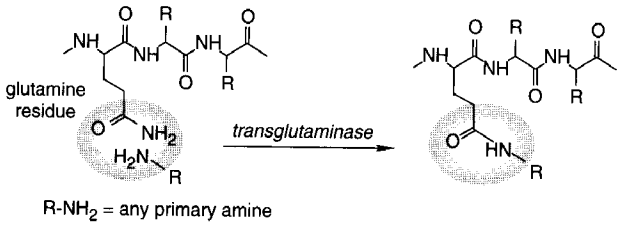

Figure 1. Transglutaminase-catalysed reaction of glutamine residues with primary amines.

amine, *o*-phosphorylethanolamine, into protein–Gln residues. Our overall goal is to be able to add various functional groups onto the Gln residues on wool fibre using TGase. RCM-κ-casein is a protein substrate for TGases.[16] Here we used reduced carboxymethylated (RCM-)κ-casein and gelatin as model systems with TGase to cross-link and/or covalently add a primary amine to the model proteins.

MATERIALS AND METHODS

Reagents

Bovine RCM-κ-casein, was prepared according to Groves *et al.*[17] Commercial 225 and 75 Bloom gelatins, type B from bovine skin, characterized at this laboratory as 225 g and 95 g Bloom (G-225 and G-95), respectively, were obtained from Sigma (St Louis, MO, USA). Worsted flannel wool fabrics (Testfabrics Inc, No 523, balanced plain weave) were purchased from Testfabrics (West Pittston, PA, USA).

Activa TG-TI, a microbial transglutaminase (100 units g^{-1}) containing maltodextrin as a carrier, with activity in the pH 4.0–9.0 range, at 0–70 °C, was obtained from Ajinomoto USA Inc (Paramus, NJ, USA). *o*-Phosphorylethanolamine, Triton X-100, polyvinyl alcohol, *N*-ethylmaleimide, malachite green–HCl, ammonium molybdate and hydrogen peroxide were purchased from Sigma (St Louis, MO, USA). Phast gels, 4–15% gradient, were purchased from Pharmacia (Hercules, CA, USA). All other reagents used were of analytical grade or ACS certified.

Model system for transglutaminase-catalysed reaction on RCM-κ-casein

Monomeric RCM-κ-casein (2.8 mg) was suspended in 1.0 ml of 0.1 M Tris–HCl (pH 8.5); 0.5 ml of the resulting solution was pre-incubated at 37 °C for 1 h. Aliquots (250 µl) of the incubated RCM-κ-casein and non-pre-incubated controls were dispensed into small test tubes (two each). To one test tube containing the pre-incubated casein and to one tube containing the control were added 50 µl of a solution containing 14 µg ml^{-1} of TGase and 50 µl of 71 mg ml^{-1} *o*-phosphorylethanolamine, both dissolved in Tris-buffer. A control tube contained the same amount of *o*-phosphorylethanolamine with no enzyme. All tubes were incubated for 3 h at 37 °C followed by heating at 90 °C for 5 min to inactivate the enzyme. The efficiency of the reaction was determined as follows: half of the catalysed reaction was saved for SDS–PAGE analysis to check for the formation of crosslinks. The other half was dialysed to remove excess amine and treated as described below to quantitate the amount of bound phosphate.

Electrophoretic analysis

SDS–PAGE was run on a Pharmacia Phast Gel system according to the method of Laemmli.[18] Samples were loaded on 4–15% gradient gels. After electrophoresis the gels were stained with Coomassie brilliant blue R-250 and destained. A flow chart of the samples run by the above method is shown in Fig 2.

Determination of phosphate bonded to κ-casein

Dialysed phosphorylated RCM-κ-Casein (100 µl of 2.4 mg ml^{-1}) was freeze-dried. HCl (600 µl of 6 M) was added to the dry flask which then was flushed with nitrogen and sealed. The treated tubes were hydrolyzed overnight at 113 °C. Analysis of the inorganic phosphate released was carried out using the malachite green assay[19] and read at 630 nm using a Varian Cary-50 spectrophotometer to detect the presence of inorganic phosphate.

Model system for transglutaminase-catalysed reaction on gelatin

Gelatins are classified by their Bloom value, a measure of their strength. Stronger gels give higher Bloom values. G-95 and G-225 (20 mg each) gelatins were suspended in 2 ml (0.1 M) Tris–HCl pH 8.5 and allowed to swell for 1 h. The resulting suspensions were heated to 60 °C for a few minutes for the particles to dissolve and then were divided into four 500 µl aliquots. To tube 1 was added 50 µl of 14 µg ml^{-1} of TGase and the primary amine *o*-phosphorylethanolamine (50 µl of 71 mg ml^{-1}); to tube 2, 50 µl of 14 µg ml^{-1} of TGase; to tube 3 the primary amine *o*-phosphorylethanolamine (50 µl of 71 mg ml^{-1}); and to the control tube 4 no additions.

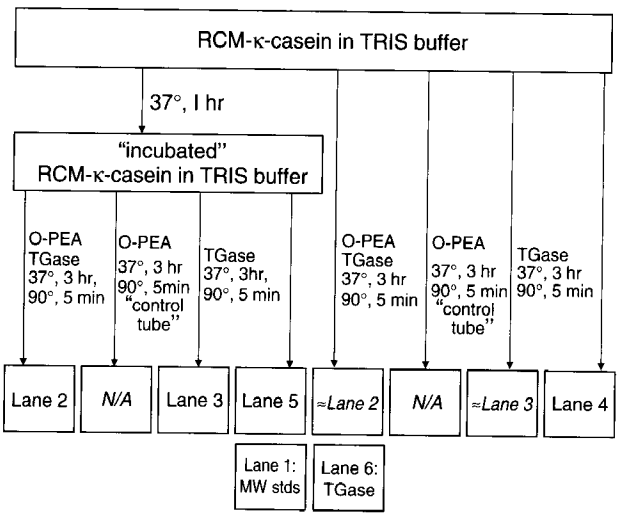

Figure 2. Variations in reaction conditions and correlation to SDS–PAGE gel lanes (Fig 3). N/A, not applicable; O-PEA, *o*-phosphorylethanolamine.

All the tubes were incubated at 40 °C for 4 h. The reaction was terminated by the addition of 1% *N*-ethylmaleimide solution. The samples were dialysed, and the bound inorganic phosphate was detected by hydrolysis and malachite green assay[19] as mentioned above. The efficiency of the reaction was also detected by SDS–PAGE analysis.

Transglutaminase-catalysed reaction on wool fabric

Four pieces of woven wool fabric, approximately 1.5 g each, were used. Fabrics were placed in individual reaction tubes containing 100 ml of 0.1 M Tris–HCl buffer, pH 8.5 and 1% Triton X-100 for 1 h before the addition of 0.015 g TGase dissolved in Tris–HCl buffer. Four concentrations of *o*-phosphorylethanolamine (35, 71, 106 and 142 mM) were used. The tubes were incubated at 40 °C for 4 h. The reaction was terminated with 1 ml of 1% *N*-ethylmaleimide solution. The fabric was rinsed five times with distilled water and vacuum-dried before further analysis. Bound phosphate was analysed using the method described below. The fabrics were also subjected to mechanical testing as described below.

Determination of phosphate in wool fabric

Approximately 0.4 g samples of phosphorylated woven wool fabric from the above experiment were each placed into a Kjeldahl flask. The fabric was digested with 1 ml of 50% sulphuric acid (v/v) and heated until the solution darkened.[20] The flask was cooled for 15 s before a few drops of 30% hydrogen peroxide were added. The mixture was again allowed to simmer for 20 min. The cycle was repeated several times with the addition of two to four drops of hydrogen peroxide until solutions turned pale yellow or colourless. Phosphate analysis on the woven wool fibre was performed as reported.[20]

Tensile strength

Mechanical property measurements were performed on the wool fabric. Measurements included tensile strength, Young's modulus and initial strain energy. Tensile strength is the maximum stress sustained under a tensile force without fracture.[21] Young's modulus is a physical quantity representing the stiffness of a material. It is determined by measuring the slope of a line tangent to the initial stress–strain curve. The initial strain energy is defined as the energy needed to stretch the fabric to 10% strain.[22] This is the area under the stress–strain curve from 0 to 10% strain. If other material variables are equal, the initial strain energy will be proportional to the volume of the tested samples. To compare different samples, the value of initial strain energy for each test sample was divided by the volume of that sample to obtain the initial strain energy with the SI unit of $J\,cm^{-3}$. These properties were measured with a gauge length (the distance between two grips) of 25.4 mm. Test samples were stored in a conditioned room at 23 °C and 65% RH before testing according to ASTM standard method D1610-01. An upgraded Instron mechanical property tester, model 1122 and Testworks 3.1 data acquisition software (MTS Systems Corp, Minneapolis, MN, USA) were used throughout this work. The strain rate (crosshead speed) was set at 300 mm min^{-1}. Because of limited sample availability, each mechanical property test was run in sets of five.

RESULTS AND DISCUSSION

Transglutaminase-catalysed reaction on κ-casein

The caseins of milk form a unique calcium-phosphate transport complex, which provides necessary nutrients to the neonate. The colloidal stability of these particles is due primarily to κ-casein. As purified from milk, this protein exhibits a unique disulphide bonding pattern, which (in the absence of reducing agents) ranges from monomer to octamers and above on SDS–PAGE. Recent studies of κ-casein have shown that, when the protein was reduced, carboxymethylated (RCM-κ-casein) and pre-incubated (tempered) for 30 min at 37 °C, the protein formed fibrillar structures instead of spherical particles.[23,24]

The fibrillar structures were up to 400 nm in length. Circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies had been used in this laboratory to investigate the temperature-induced changes in the secondary structure of the RCM-κ-casein. These studies had suggested little change in the distribution of secondary structural elements during this transition, with extended strand and β turns predominating, and previous studies on amyloid proteins have suggested that such motifs promote fibril formation.[25]

Christensen *et al*[26] studied the peptides resulting from the reaction of TGase with κ-casein in the presence of the diamine putrescine. They demonstrated that Gln residues 29, 45, 114 and 163 were good acyl donors for the reaction with putrescine. Additionally, Lys residues 21 and 24 formed either inter- or intra-molecular cross-linkages with Gln 45. It is important to note that in their studies the κ-casein was thoroughly reduced with DTT prior to incubation with the TGase at 37 °C for 3 h. The conditions used by Christensen and coworkers would have initiated the formation of fibrils noted above. The physical aggregation to fibrils could be the cause of inter-molecular cross-linkages.

In our studies, RCM-κ-casein was used to evaluate the ability of TGase to cross-link the protein. The lack of disulphide bonds in the modified protein causes it to migrate as low molecular weight species on SDS–PAGE, so the presence of cross-links induced by TGase could readily be evaluated by this method. Figure 3 shows the un-reacted enzyme in lane 6 and the non-pre-incubated RCM-κ-casein in lane 4; both migrate as essentially single bands with reduced molecular weights of 33 000 and 19 000, respectively.

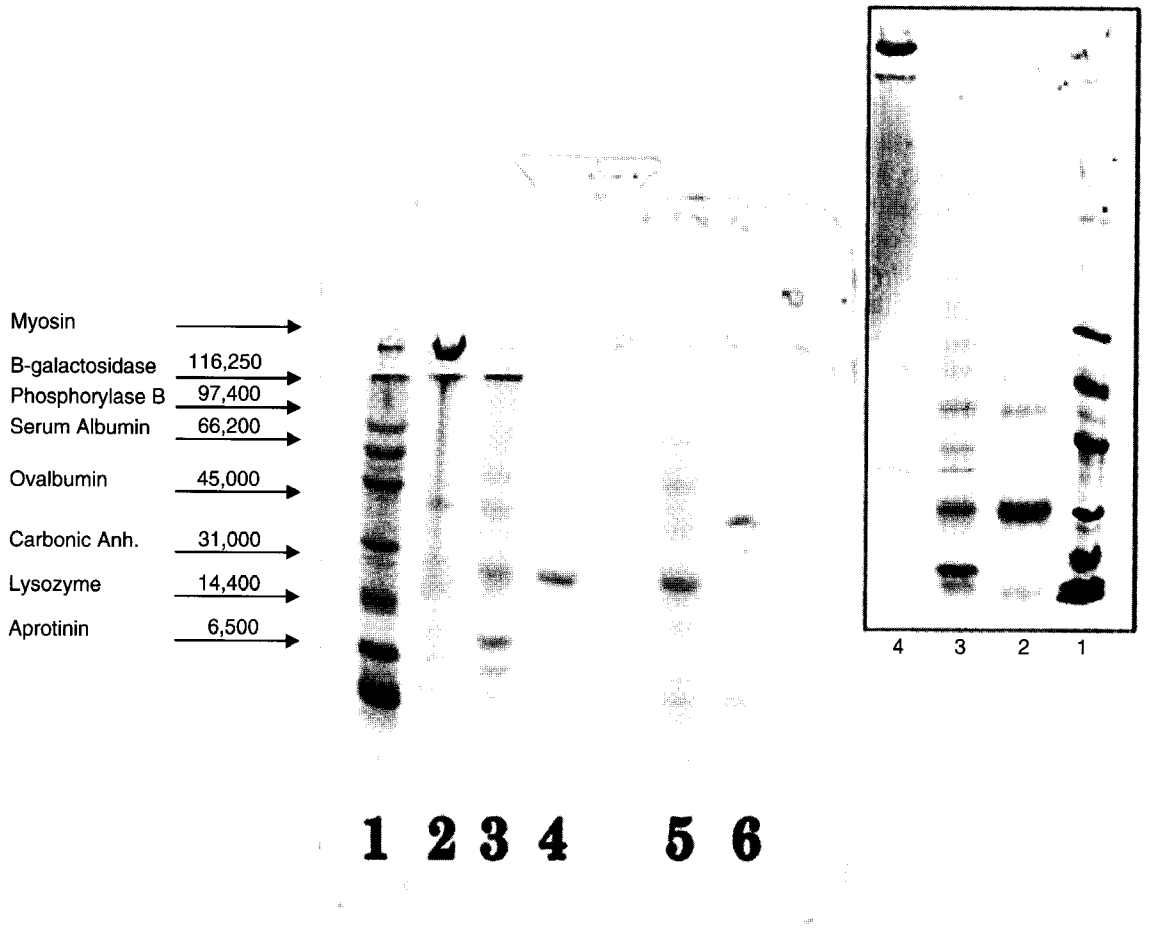

Figure 3. SDS–PAGE gel electrophoresis showing cross-linkage in RCM-κ-casein and covalent bonding with *o*-phosphorylethanolamine (O-PEA). Lane 1 shows molecular weight standards; lane 2 shows pre-incubated RCM-κ-casein, TGase and *o*-phosphorylethanolamine; lane 3 shows pre-incubated RCM-κ-casein with TGase enzyme; lane 4 shows non-pre-incubated RCM-κ-casein; lane 5 shows pre-incubated RCM-κ-casein without the enzyme TGase; lane 6 shows TGase alone. (Inset shows the molecular weight standards (lane 1) and non-preincubated RCM-κ-casein (lane 2), with enzyme TGase (lane 3) and enzyme TGase plus O-PEA (lane 4).).

Lane 5 shows pre-incubated RCM-κ-casein in the absence of any reactants; small amounts of SDS-resistant dimer and trimer are caused by the incubation at 37 °C. The reaction of RCM-κ-casein at 37 °C with the enzyme TGase alone (lane 3) causes a significant increase in higher-molecular-weight species, including polymers of >100 000, which are unable to penetrate the separating portion of the SDS–PAGE gel; these conditions are equivalent to those of Christensen *et al*,[26] who demonstrated both amine incorporation and cross-linkages. The reaction products of TGase with RCM-κ-casein that was not pre-incubated at 37 °C to promote fibril formation were identical to those seen in lane 3 (Fig 3). In this preparation of TGase, some protease may also be present, as lower molecular weight species of RCM-κ-casein are also present (lane 3). In contrast, the presence of *o*-phosphorylethanolamine in the pre-incubated reaction mixture as shown in lane 2 (and the non-preincubated reaction mixture; Fig 3 inset) led to the formation of highly cross-linked polymeric species, which, for the most part, are unable to penetrate even the low porosity SDS stacking gel (lane 2). Apparently the *o*-phosphorylethanolamine not only may be attached to the RCM-κ-casein, but also somehow leads to either increased inter-molecular cross linkages that are not DTT-sensitive, or increased protein–protein interactions that are not broken down by SDS. Figure 4 shows the amount of phosphate bound to the RCM-κ-casein.

Transglutaminase-catalysed reaction with gelatin

As reported,[2] the physical properties of gelatin can be modified with the use of enzyme under certain

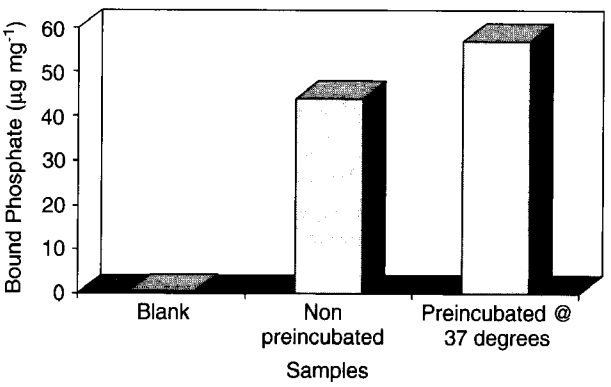

Figure 4. Amount of phosphate bound to untreated and treated RCM-κ-casein.

experimental conditions. When one to five units of the enzyme were used per gram of gelatin, the melting point and the Bloom strength increased. In the present work, G-225 and G-95 gelatin samples were reacted as described in the Materials and Methods. Although gelatins are blends of varying molecular weights and do not produce well defined bands on SDS–PAGE gels, Fig 5 shows that, in the presence of TGase, intra- and inter-molecular crosslinks led to the formation of large aggregates that could not enter the SDS–PAGE gel. Lanes that contain the enzyme or both the enzyme and *o*-phosphorylethanolamine demonstrated high-molecular-weight polymers, as the protein did not migrate through the low porosity stacking gel. Samples containing gelatin and *o*-phosphorylethanolamine alone showed no difference from the control sample (gelatin alone), showing that, if any crosslinking had occurred, it must have been minimal as it was not detected on the gel (not shown). These samples were later dialysed to remove unbound phosphate and analysed for bound phosphate using the malachite green assay (Fig 6). G-95g with *o*-phosphorylethanolamine without TGase was used as a control to confirm complete dialysis of unbound *o*-phosphorylethanolamine.

Transglutaminase-catalysed reaction on wool fabric

We investigated the covalent binding of a primary amine to wool through a TGase-catalysed reaction.

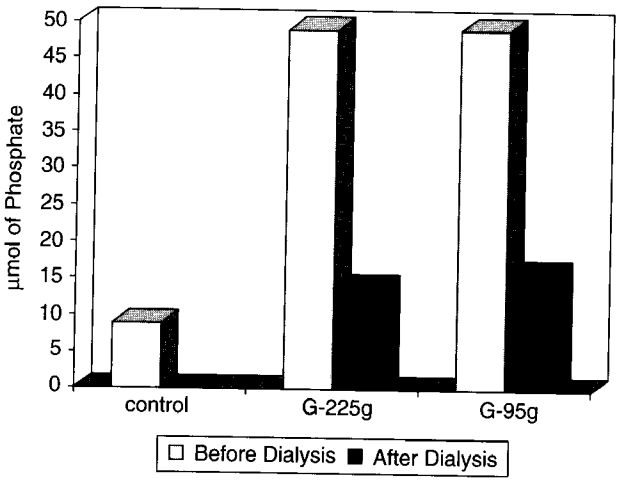

Figure 6. Amount of phosphate bound to gelatin (using malachite green assay[23]) after dialysis to release unbound phosphate. Control: G-95g gelatin plus *o*-phosphorylethanolamine, no TGase; G-225g and G-95g: gelatins plus *o*-phosphorylethanolamine plus TGase.

Wool fabric was treated with TGase as described in the materials and Methods. After several washings, approximately 0.4 g of the phosphorylated wool fabric was digested with acid and analysed for bound phosphate using the EPA method.[20] These results indicate that, after several washings, some *o*-phosphorylethanolamine is covalently bonded to the wool fibre/fabric. The data, as shown in Fig 7, indicate that *o*-phosphorylethanolamine is covalently bonded

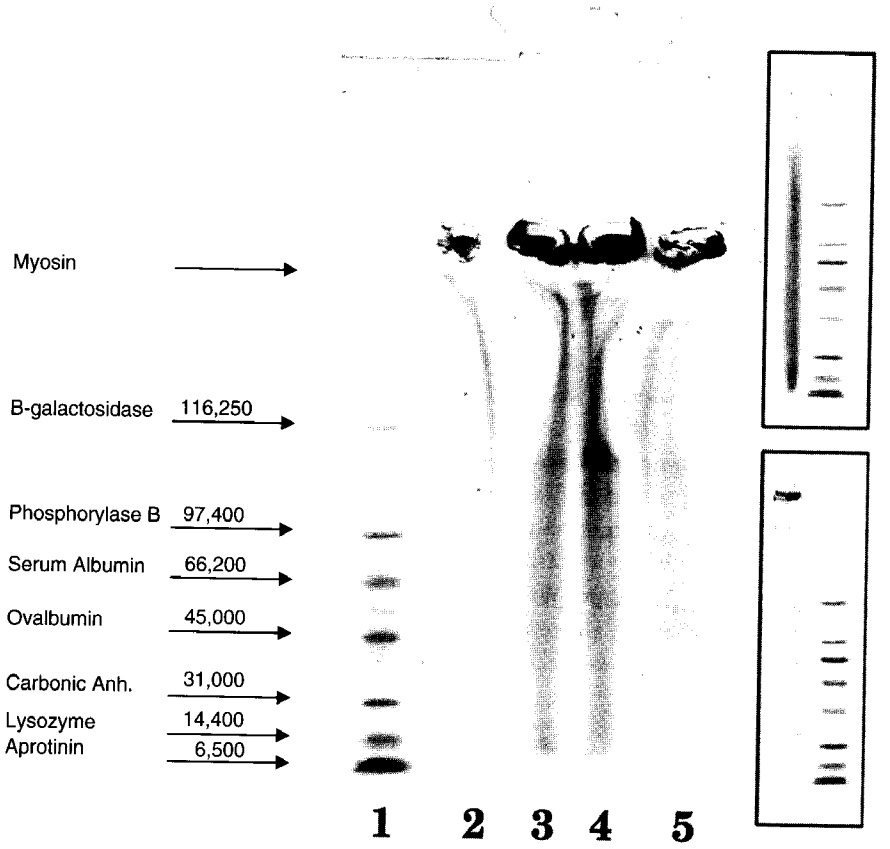

Figure 5. SDS–PAGE showing the cross-linkage and covalent bonding with gelatin. Lane 1 shows molecular weight standards; lane 2 shows G-225g with TGase; lane 3 shows G-225g with TGase and *o*-phosphorylethanolamine; lane 4 shows G-95g with the enzyme TGase; lane 5 shows G-95g with the enzyme TGase and *o*-phosphorylethanolamine. (Insets show molecular weight standards and gelatin with two different Bloom values: top inset G-225g and lower inset G-95g.).

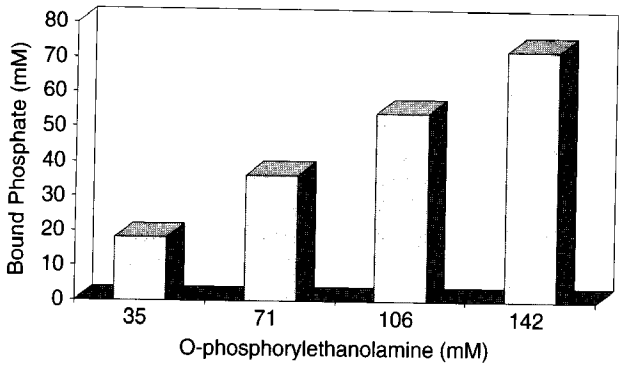

Figure 7. Amount of phosphate bound to wool fiber using EPA method 365.3[24] as a function of *o*-phosphorylethanolamine charge (35, 71, 106 and 142 mM, respectively) in the reaction with 0.4 g wool.

to the wool fibre/fabric since it survived four washings. For the wool fibres, the reaction is concentration-dependent, with a final concentration of 142 mM of *o*-phosphorylethanolamine being most effective (Fig 7).

Tensile strength

Tensile strength can be defined as the measure of a steady force that is necessary to break a fibre and it is usually expressed experimentally as the maximum load developed in tensile test.[26] There are many different ways of reporting breakage resistance of fabrics, such as by force, elongation and energy necessary. In Fig 8 are the tensile strength measurements of the *o*-phosphorylethanolamine-treated fabric before and after treatments. The tensile strength of the control fabric (no TGase, and *o*-phosphorylethanolamine) overall did not show a major change with the increase of *o*-phosphorylethanolamine in the two samples used. However, in Fig 9, the elongation property increased with increased levels of *o*-phosphorylethanolamine. The enzymatic addition of *o*-phosphorylethanolamine did not affect the tensile strength of the fabric, but the elongation property was dramatically increased.

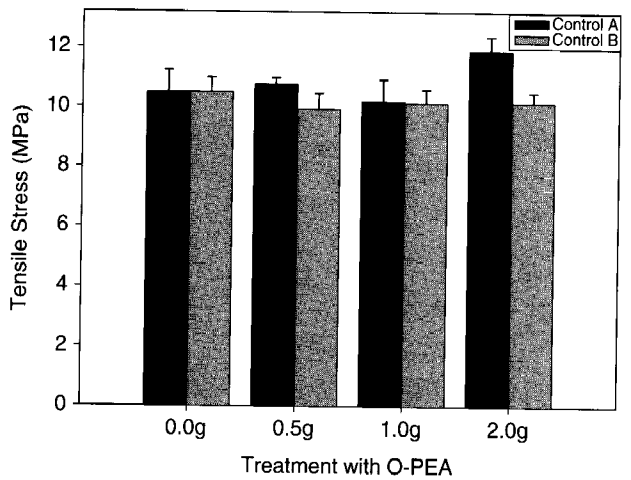

Figure 8. Tensile strength of woven fabric with TGase and *o*-phosphorylethanolamine.

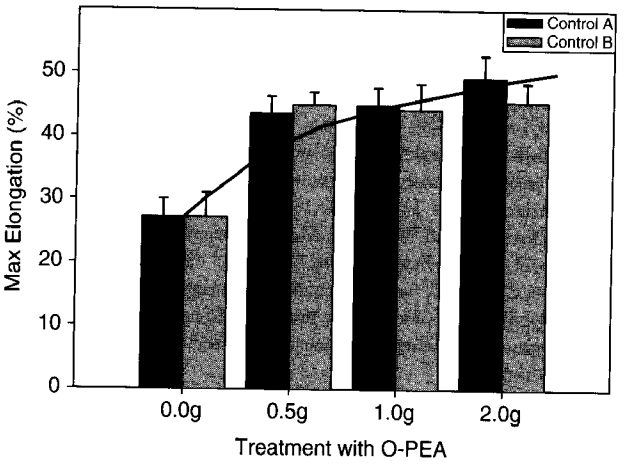

Figure 9. Elongation property of woven fabric.

CONCLUSIONS

In this study, we examined TGase-catalysed crosslinking and covalent bonding on two soluble proteins and on wool fiber. We provided evidence that TGase produced inter- and intra-molecular crosslinks in the proteins used and also catalysed the covalent bonding of *o*-phosphorylethanolamine to RCM-κ-casein, gelatin and wool. The preliminary results using TGase with the fibre are encouraging as the treated wool was whiter with a softer feel than the untreated fibre. We are conducting further studies to determine the treatment effect on wool's mechanical and flame-retardant properties and also on the tensile strength of the treated fibres.

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Title: Studies to Select Appropriate Nonpathogenic Surrogate *Escherichia coli* Strains for Potential Use in Place of *Escherichia coli* O157:H7 and *Salmonella* in Pilot Plant Studies

Author(s): D. R. Eblen, B.A. Annous, and G.M. Sapers

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Studies To Select Appropriate Nonpathogenic Surrogate *Escherichia coli* Strains for Potential Use in Place of *Escherichia coli* O157:H7 and *Salmonella* in Pilot Plant Studies†

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ABSTRACT

The response of a potential nonpathogenic surrogate organism to a particular treatment should closely mimic the response of the target pathogenic organism. In this study, growth characteristics (generation time, lag phase duration, and maximum population), pH at stationary phase, and survival characteristics (level of attachment and survival on apple surfaces, resistance to hydrogen peroxide decontamination treatments, and thermal resistance at [illegible]) of 15 nonpathogenic generic *Escherichia coli* strains and one nonpathogenic *E. coli* O157:H43 strain were compared with those of four *E. coli* O157:H7 strains and four *Salmonella* strains. Few differences in growth characteristics or pH at stationary phase were observed between nonpathogenic and pathogenic strains tested. However, considerably more separation among strains was seen following investigation of survival characteristics. *E. coli* ECRC 97.0152, which does not contain genes encoding for known virulence factors associated with *E. coli* O157:H7, appears to be a good surrogate candidate, with growth and survival characteristics similar to those of *E. coli* O157:H7 strains. The heat-tolerant surrogate strains *E. coli* NRRL B-766 and NRRL B-3054 and *E. coli* ATCC 11775, ATCC 23325, and ATCC 25922 may be used when attempting to model the heat resistance of *Salmonella* Montevideo G4639 and *Salmonella* Poona RM 2350, respectively. These surrogate strains may be useful for evaluating the efficacy of intervention steps in reducing populations of inoculated strains of *E. coli* O157:H7 and *Salmonella* in processing environments where these pathogens cannot be introduced.

Researchers in the field of applied microbial food safety have many factors to consider when designing validation and challenge studies. Often, the goal of such experiments is to characterize the behavior of a particular pathogen in a target food or food processing step with a view to developing a way of eliminating or controlling pathogen proliferation. Although the use of the relevant target pathogen would be ideal, researcher safety concerns must also be considered. In many instances, researchers find it necessary to use a nonpathogenic surrogate organism in place of the target pathogenic organism. Such studies are inherently limited because surrogate organisms may not behave in a manner identical to that of their pathogenic counterparts. Nonpathogenic surrogate organisms do not have the virulence genes of wild-type outbreak strains, i.e., the type of strains whose behavior and responses the research is attempting to reproduce. Pathogenic strains also may have unique characteristics that contribute to their virulence. For example, *Escherichia coli* O157:H7 is highly tolerant of acidic conditions ([illegible]), a factor that has been cited as contributing to the virulence of this pathogen by permitting survival at the low pH of the stomach ([illegible]). The U.S. Food and Drug Administration ([illegible]) defines a surrogate microbe as "a nonpathogenic species and strain responding to a particular treatment in a manner equivalent to a pathogenic species and strain. The surrogate allows biological verification of the treatment without introducing pathogens into a food processing area." Therefore, potential nonpathogenic surrogate organisms should be characterized prior to use in validation and challenge studies and should be negative for virulence factors of target pathogens relevant to the food system of interest.

Our laboratory is engaged in pilot plant studies to characterize the efficacy of various washing and sanitizing treatments in reducing populations of pathogenic microflora, specifically *E. coli* O157:H7 and *Salmonella*, on fresh fruit and vegetables. Because these organisms cannot be introduced to the pilot plant in its present configuration because of concerns for the safety of equipment operators or other personnel in the pilot plant area, nonpathogenic organisms are needed. The aim of the present study was to match the growth, survival, and thermal resistance characteristics of 15 nonpathogenic generic *E. coli* strains and one nonvirulent (nontoxigenic) O157:H43 *E. coli* strain with characteristics of four strains each of *E. coli* O157:H7 and *Salmonella*. All four pathogenic strains were previously associated with foodborne outbreaks in produce or apple cider. Growth response and thermal inactivation of bacteria were previously shown

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† Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

‡ Annous and Eblen contributed equally to this work and are considered as senior authors.

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TABLE 1. *Bacterial strains*

Strain[a]	Source
E. coli O157:H43[b]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 96.0509[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 97.0147[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 97.0152[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 97.0190[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 99.0512[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ECRC 99.1232[c]	Dr. D. Debroy, Penn State University, University Park, Pa.
E. coli ATCC 11775[d]	Dr. P. Fratamico, USDA-ARS-ERRC, Wyndmoor, Pa.
E. coli ATCC 25253	Dr. R. Merker, FDA-CFSAN, Washington, D.C.
E. coli ATCC 35695	Dr. R. Merker, FDA-CFSAN, Washington, D.C.
E. coli ATCC 25922	Dr. W. Fett, USDA-ARS-ERRC, Wyndmoor, Pa.
E. coli NRRL B-766	Dr. L. K. Nakamura, USDA, National Center for Agricultural Utilization Research (NCAUR), Peoria, Ill.
E. coli NRRL B-2783	Dr. L. K. Nakamura, USDA, NCAUR, Peoria, Ill.
E. coli NRRL B-3054	Dr. L. K. Nakamura, USDA, NCAUR, Peoria, Ill.
E. coli NRRL B-3704	Dr. L. K. Nakamura, USDA, NCAUR, Peoria, Ill.
E. coli NRRL B-14573	Dr. P. Fratamico, cider outbreak strain
E. coli O57:H7 SEA 13B88	Dr. M. Lytle, Oklahoma State Department of Health, Oklahoma City; cider outbreak strain
E. coli O157:H7 OK	Dr. W. Fert, cantaloupe outbreak strain
Salmonella Poona RM 2350	Dr. L. Beuchat, University of Georgia, Griffin; tomato outbreak strain
Salmonella Montevideo G4639	

[a] ECRC, *E. coli* Reference Center, University Park, Pa.; ATCC, American Type Culture Collection; NRRL, Northern Regional Research Laboratory, Peoria, Ill.
[b] Nonpathogenic strain.
[c] All O55 strains.
[d] Contains green fluorescent protein on pGFPuv plasmid.

to be dependent on environmental parameters such as the growth medium *(2, 3, 5)*. In this study, the growth profiles and thermal resistance of all strains were examined following incubation in five typical growth media. Ongoing studies at our laboratory concern novel methods of decontaminating fruit with H_2O_2 and other antimicrobial agents; therefore, the level of attachment of the test strains to apple surfaces and their subsequent reduction by washing with a H_2O_2 solution were also investigated. Our goal was to identify a nonpathogenic surrogate that would not pose a health hazard and whose behavior closely resembled that of the pathogens represented under the parameters examined.

MATERIALS AND METHODS

Strains. Fifteen non-O157:H7 *E. coli* strains, one nonpathogenic *E. coli* O157:H43 strain, two *E. coli* 0157:H7 outbreak strains, and two *Salmonella* outbreak strains were used (Table 1). *E. coli* O157:H43, a nonpathogenic mutant of O157:H7, was included for comparison purposes only and was not considered as a possible surrogate. All strains were stored in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) containing 20% (vol/vol) glycerol at −80°C. Working stocks were kept on tryptic soy agar (TSA; Difco, Becton Dickinson) slants containing 0.6% (wt/vol) yeast extract (Difco, Becton Dickinson) at 4°C for 2 weeks.

All *E. coli* strains were confirmed by streaking on eosin methylene blue agar (Difco, Becton Dickinson) on which *E. coli* produces a green sheen with reflected light. *E. coli* O157:H7 and O157:H43 colonies from each study were further identified using the RIM *E. coli* O157:H7 latex agglutination test (Remel, Lenexa, Kans.), which identifies the O157 and H7 antigens. *Salmonella* strains were confirmed using a commercial latex agglutination test (Oxoid, Basingstoke, UK), which identifies the flagellar antigens.

All potential surrogate strains included in the study were submitted to the Gastroenteric Disease Center (Wiley Lab, Pennsylvania State University, University Park, Pa.) and tested for the presence of genes encoding for known virulence factors associated with *E. coli* O157:H7: (i) STa, heat stable toxin; (ii) STb, heat stable toxin; (iii) LT, heat labile toxin; (iv) SLT-I, Shiga-like toxin I; (iv) SLT-II, Shiga-like toxin II; (v) K99, fimbrial adhesion factor; (vi) *eae*, attaching and effacing gene; (vii) F1845, fimbrial gene; and (viii) CS31A, attachment factor.

Preparation of growth media. Five different growth media were prepared according to the manufacturers' instructions: (i) TSB (standard formulation contains 0.25% glucose); (ii) TSB+G (TSB supplemented with glucose [Sigma; St. Louis, Mo.] to a final concentration of 1% [wt/vol]); (iii) TSB−G (TSB with no glucose; Difco, Becton Dickinson); (iv) TSB−GHCl (TSB−G adjusted to pH 5.0 with 0.1 M HCl); and (v) brain heart infusion broth (BHI; Difco, Becton Dickinson). Initial pH of uninoculated TSB, TSB−G, TSB+G, and BHI was 7.4 to 7.6. BHI and TSB were chosen as typical growth media. TSB+G was included because it has been reported that the acid tolerance of *E. coli* O157:H7 is enhanced during growth in the presence of glucose in this manner because of adaptation to gradual production of acid by the metabolizing cells *(6)*. However, such growth conditions can result in a significant amount of injury to growing cells *(6, 14)*, which may impair their subsequent responses to stress. Therefore, the magnitude of the effect of glucose and acidity was investigated by growing the cells in TSB that contained no glucose (TSB−G) and by growing the cells in TSB−G in which HCl (an inorganic acid) was present as the acidulent (TSB−GHCl). The pH of each

broth was measured in triplicate after preparation and after cell growth of each strain reached the stationary phase.

Growth characteristics. Individual strains were grown in 10 ml of TSB at 37°C for 16 h with shaking at 75 rpm. The active culture was then diluted 1:100 with sterile 0.1% (wt/vol) peptone water (PW; Difco, Becton Dickinson), and 1 μl of this dilution was transferred to triplicate 30-ml volumes of each of the five growth media (TSB, TSB+G, TSB−G, TSB−GHCl, and BHI) in 250-ml Erlenmeyer flasks. The flasks were incubated at 37°C with shaking at 75 rpm. Populations were enumerated by aseptically removing a 0.1-ml aliquot of the culture at 0, 1, 2, 4, 6, 8, 10, and 24 h, diluting in 0.1% (wt/vol) PW as necessary, and surface plating on TSA. TSA plates were incubated overnight at 37°C prior to counting. Growth curves were generated by fitting the Gompertz function to the data *(21)*. The Gompertz function parameters were then used to calculate the generation time, lag phase duration, and maximum population density (log CFU per milliliter) for each strain in each growth medium.

Attachment and survival on apple surfaces and resistance to H_2O_2 wash treatments. Individual strains were grown in 10 ml of TSB at 37°C for 8 h and used to inoculate 1 liter of TSB at 0.01% (vol/vol) concentration, which was then incubated at 37°C for 16 h with shaking at 75 rpm. Cells were recovered by centrifugation at 11,170 × *g* for 10 min at 4°C, washed once with 250 ml of PW, and suspended in 2 liters of sterile distilled water. This cell suspension was used to inoculate apples. Initial cell populations were determined by serially diluting the culture in PW and surface plating duplicate 0.1-ml aliquots on TSA.

Unwaxed Golden Delicious apples (32 per experiment) obtained from a single Washington State grower were removed from storage at 4°C and inoculated in less than 1 h with the strain under investigation by submerging apples (six at once) in 2 liters of inoculum at 20°C for 5 min. The apples were placed on their sides to allow for drainage from the stem and calyx areas and left to air dry for 2 h on absorbent paper on the bench top. At this point, half of the apples (n = 16) were stored at 4°C for 24 h. The remaining apples were divided into two sets of eight. The first set was divided into duplicate composite sets of four apples. Each apple was weighed and cut into quarters using a sterile knife and cutting board. Apple pieces of each composite set were placed in a 4-liter stainless steel blender (Waring, Torrington, Conn.) with a volume of PW equal to the apple weight and blended on low speed for 1 min. The resulting blend was filtered through a filter stomacher bag (Spiral Biotech, Bethesda, Md.), and duplicate 10-ml volumes were transferred to sterile tubes. This procedure was repeated with the second composite set of fruit. The remaining eight apples were separated into two sets of four apples, and each set of apples was washed with 5% (vol/vol) H_2O_2 solution at room temperature (prepared from a 30% [vol/vol] H_2O_2 solution; Fisher Scientific, Pittsburgh, Pa.) with shaking for 1 min, briefly drained, and immediately blended. The entire procedure was repeated for apples stored for 24 h. Filtrates were diluted in PW as necessary and surface plated on the appropriate enumeration agar medium. Uninjured cells of nonpathogenic *E. coli*, *E. coli* O157:H7, and *Salmonella* were enumerated on selective MacConkey agar (Difco, Becton Dickinson), sorbitol MacConkey agar (Difco, Becton Dickinson), and xylose lysine Tergitol 4 agar (Difco, Becton Dickinson), respectively. Recovery medium (TSA) was used for enumeration of injured *E. coli* and *Salmonella* cells. After inoculation, TSA plates were incubated at 37°C for 2 h to allow recovery of injured cells and were then overlaid with the appropriate selective medium *(10)*. All plates were incubated overnight at 37°C, and resultant colonies were manually counted.

Thermal inactivation. The D-value (time to reduce bacterial populations by 1 log or 90%) of each organism under investigation was determined at 60°C ($D_{60°C}$). Individual strains were grown in 10 ml of TSB at 37°C for 8 h with shaking at 75 rpm and were used to inoculate 30 ml of each of the five growth media (TSB, TSB+G, TSB−G, TSB−GHCl, and BHI) at 0.01% (vol/vol) concentration. Cells were grown for 16 h with shaking at 75 rpm at 37°C, recovered by centrifugation, washed once with PW, and suspended in 30 ml of sterile PW. Samples with similar cell densities were heat treated at 60°C by the method of Cole and Jones *(7)*, using a Techne submerged-coil heating apparatus model tempette TE-8D (Protocol Instruments Ltd., West Byfleet, UK). Each thermal inactivation procedure was performed in triplicate. Heat-treated samples were serially diluted in PW, and duplicate 0.1-ml aliquots were surface plated on TSA. Plates were incubated overnight at 37°C prior to counting. The D-value was calculated by plotting log number of survivors against time and obtaining the reciprocal of the slope of the line using Excel spreadsheet software (Microsoft Corporation, Redmond, Wash.).

Statistical analyses. Analyses of variance with individual contrasts and Bonferroni t tests were performed to determine significant differences between population means in response to strain and medium and to determine medium-strain interactions. Comparisons of final pH values, growth parameters, attachment and resistance to H_2O_2 wash, and thermal inactivation for each strain in each medium were made using the Bonferroni least significant difference separation technique. All statistical analyses and calculations of means and standard deviations were performed using SAS/STAT software (SAS Institute Inc., Cary, N.C.).

RESULTS

Nonpathogenicity confirmatory tests. All nonpathogenic *E. coli* strains included in the study were tested for the presence of virulence genes associated with *E. coli* O157:H7. All nonpathogenic *E. coli* strains were negative for all virulence genes tested, with the exception of strain NRRL B-2783, which was positive for the O157 gene. Consequently, this strain and the O157:H43 strain were not considered as potential surrogates in the final analysis.

Changes in broth pH following microbial growth. The pH values of uninoculated broths incubated as controls decreased by 0.04 to 0.11 units following incubation overnight at 37°C. Every strain, unless otherwise noted, after being grown in broth exhibited the following relationship of final pH: TSB−G > BHI = TSB > TSB−GHCl > TSB+G ($P < 0.05$) (Table 2). An exception to this pattern was seen with *E. coli* NRRL B-14573, where a lower pH was obtained after growth in TSB−GHCl compared with growth in TSB+G, and no difference was observed in final pH between TSB and TSB+G (data not shown). No significant difference in final pH ($P < 0.05$) was noted for TSB−GHCl and BHI after growth of *E. coli* ECRC 96.0509 (data not shown). For *Salmonella* Poona, there was no difference in the final pH ($P < 0.05$) after growth in BHI, TSB, and TSB−GHCl, whereas for *Salmonella* Montevideo growth in BHI resulted in a significantly higher final pH ($P < 0.05$) than growth in TSB and TSB−GHCl, whose final pH did not differ significantly ($P < 0.05$) (Table 2).

Table 2 lists the potential surrogate strains with final

TABLE 2. *Surrogate strains with significantly different ($P < 0.05$) final pH values compared with pathogenic strains*[a]

Pathogenic strain	Growth medium: BHI		TSB		TSB+G		TSB−G		TSB−GHCl	
	Surrogate strain	pH[b]	Surrogate strain	pH	Surrogate strain	pH	Surrogate strain	pH	Surrogate strain	pH
E. coli O157:H7 SEA 13B88	96.0509[c]	6.00 ± 0.03	NS[d]	5.97 ± 0.05	B-14573[e]	4.77 ± 0.01	B-14573[e]	7.10 ± 0.03	NS	5.45 ± 0.02
E. coli O157:H7 OK	96.0509[c]	6.00 ± 0.04	NS	5.96 ± 0.03	B-14573[e]	4.74 ± 0.02	NS	7.09 ± 0.03	NS	5.41 ± 0.03
Salmonella Montevideo G4639	SL[f]	6.56 ± 0.86	B-14573[e] 96.0509[c]	6.08 ± 0.06	B-14573[e]	4.70 ± 0.06	25253[c]	7.35 ± 0.04	SL	5.93 ± 0.06
Salmonella Poona RM 2350	96.0509[c]	5.98 ± 0.04	B-14573[e]	6.07 ± 0.06	B-14573[e]	4.71 ± 0.02	NS	7.35 ± 0.02	SL	5.86 ± 0.06

[a] All *E. coli* strains, identified by strain numbers (see Table 1).
[b] Mean ± standard deviation.
[c] Significantly lower final pH ($P < 0.05$) than that of pathogenic strain.
[d] NS, no significant difference ($P < 0.05$) in final pH values between surrogate strains tested (Table 1) and the pathogenic strain.
[e] Significantly higher final pH ($P < 0.05$) than that of pathogenic strain.
[f] SL, all surrogate strains tested (Table 1) had significantly lower final pH ($P < 0.05$) than that of the pathogenic strain.

pH values significantly different ($P < 0.05$) from those of pathogenic strains following growth in each medium. Little separation was evident, with some exceptions. Both *Salmonella* strains had significantly higher final pH values ($P < 0.05$) than did *E. coli* O157:H7 strains in TSB−GHCl. All surrogate strains tested had a significantly lower final pH ($P < 0.05$) than that of *Salmonella* Montevideo G4639 after growth in BHI or TSB−GHCl. All surrogate strains tested had a significantly lower final pH ($P < 0.05$) than that of *Salmonella* Poona RM 2350 after growth in TSB−GHCl. The final pH of all surrogate strains grown in TSB−G was not significantly different ($P < 0.05$) from those of *Salmonella* Poona RM 2350. *E. coli* NRRL B-14573 grown in TSB or TSB+G had significantly lower and higher ($P < 0.05$) final pH, respectively, compared with both *Salmonella* strains. *E. coli* ECRC 96.0509 had a significantly lower final pH ($P < 0.05$) than did *Salmonella* Montevideo G4639 after growth in TSB and *Salmonella* Poona RM 2350 after growth in BHI. *E. coli* ATCC 25253 had a significantly lower final pH ($P < 0.05$) than did *Salmonella* Montevideo G4639 after growth in TSB−G. The final pH of both *E. coli* O157:H7 strains was not significantly different from that of the other microorganisms tested, with the following exceptions: *E. coli* ECRC 96.0509 had a significantly lower final pH ($P < 0.05$) in BHI and *E. coli* NRRL B-14573 had a significantly higher final pH ($P < 0.05$) in TSB+G. The latter strain also had a significantly higher final pH ($P < 0.05$) after growth in TSB−G than did *E. coli* O157:H7 SEA 13B88.

Growth characteristics. The analysis of three growth parameters (generation time, lag phase duration, and maximum population) was performed to determine the effects of strains and growth media. Generation times for the potential surrogate strains that were significantly different ($P < 0.05$) from pathogenic strains are shown in Table 3. Overall, there was no significant difference in generation times for all strains grown in BHI, TSB, TSB+G, and TSB−G, with the exception of *E. coli* NRRL B-14573 grown in BHI or TSB−G, which had a significant longer generation time ($P < 0.05$). Significantly longer generation times ($P < 0.05$) were seen when strains were grown in TSB−GHCl than when they were grown in the other broths tested, with the exception of *E. coli* ECRC 99.1232. Overall, *E. coli* ECRC 99.0512 grown in TSB−GHCl had the shortest generation time (0.09 h) among strains and media tested ($P < 0.05$). There was no significant difference in lag phase duration between the strains in any of the media (range, 1.56 to 2.55 h; data not shown). Significantly longer lag phases ($P < 0.05$) were seen when strains were grown in TSB−GHCl than when they were grown in the other broths tested (data not shown). Overall, the highest and lowest maximum population densities of all bacterial strains tested were obtained following growth in BHI and TSB and in TSB−GHCl, respectively (data not shown). Exceptions were the *E. coli* NRRL B-2783, NRRL B-3704, and NRRL B-766 strains, where the maximum population densities were obtained following growth in TSB−GHCl (data not shown). These population densities were not significantly

TABLE 3. *Surrogate strains with significantly different (P < 0.05) generation times compared with pathogenic strains grown in different growth media*

Strain	Generation time (h)[a]				
	BHI	TSB	TSB+G	TSB−G	TSB−GHCl
Pathogenic					
E. coli O157:H7 SEA 13B88	0.24 ± 0.02 A	0.25 ± 0.04 A	0.25 ± 0.03 A	0.24 ± 0.02 A	0.47 ± 0.09 A
E. coli O157:H7 OK	0.21 ± 0.04 A	0.24 ± 0.02 A	0.24 ± 0.03 A	0.21 ± 0.04 A	0.48 ± 0.02 A
Salmonella Montevideo G4639	0.28 ± 0.04 A	0.27 ± 0.02 A	0.17 ± 0.03 A	0.28 ± 0.04 A	0.49 ± 0.05 A
Salmonella Poona RM 2350	0.23 ± 0.03 A	0.21 ± 0.03 A	0.21 ± 0.02 A	0.23 ± 0.03 A	0.41 ± 0.08 A
Surrogate[b]					
E. coli ATCC 25253	NS[c]	NS	NS	NS	0.62 ± 0.02 B
E. coli NRRL B-3704	NS	NS	NS	NS	0.57 ± 0.05 B
E. coli NRRL B-14573	0.43 ± 0.03 B	NS	NS	0.43 ± 0.03 B	0.64 ± 0.07 B
E. coli ECRC 99.0512	NS	NS	NS	NS	0.09 ± 0.11 C
E. coli ECRC 99.1232	NS	NS	NS	NS	0.27 ± 0.04 D

[a] Each value represents the mean ± standard deviation of three trials. Means within each column with the same letter are not significantly different ($P < 0.05$).

[b] Surrogate strains not listed (see Table 1) showed no significant difference ($P > 0.05$) in generation time as compared with the pathogenic strains.

[c] NS, data not shown because values were not significantly different ($P > 0.05$) from generation times for the pathogenic strains.

different ($P < 0.05$) from each other nor was there any difference among the maximum population densities achieved in the other growth broths (data not shown). Overall, the growth characteristics of the pathogens were not significantly different from each other or, with few exceptions, from those of the potential surrogates.

Attachment and survival on apple surfaces and resistance to H_2O_2 wash treatments. Cell counts from treated apples were determined on recovery and selective media. Cell counts on recovery media were generally higher than counts on selective media, although significantly so only for potential surrogate *E. coli* strains ATCC 11775, ATCC 35695, NRRL B-14573, NRRL B-3054, NRRL B-3704, NRRL B-766, and ECRC 99.1232 and *Salmonella* Poona RM 2350 and *Salmonella* Montevideo G4639 ($P < 0.05$).

Table 4 lists the potential surrogate strains that had equivalent or greater attachment and survival ($P < 0.05$) on apple surfaces than did each pathogenic strain, with and without the H_2O_2 wash treatment, on day 0 and day 1. There were significant interactions between the days on which survivors were enumerated and whether or not the apples were treated with the 5% H_2O_2 wash ($P < 0.05$). Overall, samples from treated and untreated apples showed significantly higher counts on day 0 than on day 1 ($P < 0.05$). Reductions in cell numbers following 5% H_2O_2 wash treatment were only significant ($P < 0.05$) for apples sampled on day 0.

Thermal inactivation. Survivor curves demonstrated a linear decrease in cell numbers with time during heating at 60°C (curves not shown). Table 5 lists potential surrogate strains that had equivalent or greater $D_{60°C}$-values ($P < 0.05$) than did pathogenic strains. There was a significant interaction between the strains tested and the growth medium used ($P < 0.05$). The $D_{60°C}$-value of *E. coli* O157:H7 SEA 13B88 after growth in TSB+G was significantly higher ($P < 0.05$) than values obtained following growth in other growth media tested. Both *E. coli* O157:H7 strains were generally more thermal resistant than *Salmonella* strains ($P < 0.05$). However, there were two exceptions to this trend; following growth in TSB−G and in TSB−GHCl, *Salmonella* Montevideo G4639 did not significantly differ from *E. coli* O157:H7 SEA 13B88 ($P < 0.05$).

DISCUSSION

This study was a first attempt to match the growth characteristics, thermal resistance, attachment to produce, and resistance to a sanitizer wash treatment of nonpathogenic surrogate organisms to those of pathogenic counterparts. The lag phase duration observed for *E. coli* O157:H7 SEA 13B88 was considerably (28.6-fold) shorter than that reported by Whiting and Golden *(22)* for the same strain. These researchers grew the culture at 15°C in BHI containing 1.5% NaCl (pH 5.3), which could explain the longer lag phase duration. The final pH values for *E. coli* O157:H7 SEA 13B88 after growth in TSB+G and TSB−G (Table 2) were in agreement with those previously reported by Buchanan and Edelson *(6)*. Investigation of growth characteristics and final pH at stationary phase gave an indication of the metabolic behavior of each potential surrogate in each broth compared with the pathogenic strains. Very few differences were seen, which was not surprising given that all the strains tested are members of the *Enterobactericeae* and as such have similar responses to such test conditions, which are not inherently stressful. Therefore, any of the nonpathogenic *E. coli* strains tested had the potential to act as a surrogate organism for pathogenic strains tested with respect to growth characteristics and final pH value under these conditions. Considerably more separation among strains was seen following investigation of attach-

TABLE 4. *Surrogate strains that demonstrate attachment and survival equivalent to or greater than that of the pathogenic strains ($P < 0.05$) with and without the 5% H_2O_2 washing treatment on days 0 and 1*[a]

Pathogenic strain	Day 0		Day 1	
	Control	Washed	Control	Washed
E. coli O157:H7 SEA 13B88	(4.67 ± 0.66) A B-14573, 25253, 25922, B-2783,[b] 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232, O157:H43[b]	(3.15 ± 0.29) B 11775, B-14573, 25253, 25922, B-2783, B-3054, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152,[c] 97.0190, 99.0512, 99.1232, O157:H43	(4.28 ± 0.41) A B-14573, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232	(2.89 ± 0.62) B 11775, B-14573,[c] 25253, 25922, B-2783, B-3054, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152,[c] 97.0190, 99.0512, 99.1232
E. coli O157:H7 OK	(4.70 ± 0.48) A B-14573, 25253, B-2783, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232, O157:H43	(3.52 ± 0.26) B B-14573, 25253, B-2783, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512	(3.73 ± 0.30) B B-14573, 25253, 25922, B-2783, 96.0509, 97.0147, 97.0152,[c] 97.0190, 99.0512, 99.1232	(3.07 ± 0.42) B B-14573,[c] 25253, 25922, B-2783, B-3054, 35695, B-3704, B-766, 97.0147, 97.0152,[c] 97.0190, 99.0512, 99.1232
Salmonella Montevideo G4639	(4.89 ± 0.28) A B-14573, 25253, B-2783, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, O157:H43	(3.60 ± 0.54) B B-14573, 25253, B-2783, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512	(3.80 ± 0.60) B B-14573, 25253, 25922, B-2783, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232	(3.28 ± 0.79) B B-14573, 25922, B-766, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232
Salmonella Poona RM 2350	(4.87 ± 0.31) A B-14573, 25253, B-2783, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, O157:H43	(3.71 ± 0.65) B B-2783, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512	(3.38 ± 0.75) BC B-14573,[c] 25253, 25922, B-2783, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152,[c] 97.0190,[c] 99.0512,[c] 99.1232	(2.66 ± 0.84) C 11775, B-14573, 25253, 25922, B-2783, B-3054, 35695, B-3704, B-766, 96.0509, 97.0147, 97.0152,[c] 97.0190,[c] 99.0512,[c] 99.1232

[a] All *E. coli* strains, identified by strain numbers (see Table 1). Values in parentheses are the mean ± standard deviation (log CFU per gram) at each sampling point. Means within same row with the same letter are not significantly different ($P > 0.05$).

[b] *E. coli* NRRL B-2783 contains the O157 gene, and O157:H43 strains were not considered potential surrogates. These strains are included for comparison purposes only.

[c] Strains that had significantly greater ($P < 0.05$) attachment and survival than did the pathogenic strain.

TABLE 5. *Surrogate strains that demonstrate heat resistance ($D_{60°C}$) equivalent to or greater than that of the pathogenic strains*[a]

Pathogenic strain	Growth medium				
	BHI	TSB	TSB+G	TSB−G	TSB−GHCl
E. coli O157:H7 SEA 13B88	(65.99 ± 9.20)	(72.10 ± 2.62)	(90.07 ± 4.01)	(57.94 ± 12.40)	(58.72 ± 5.90)
	B-14573,[b] 25922, B-2783,[b,c] B-3054, 35695,[b] B-3704, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232[b] 157:H43[c]	B-14573, B-2783,[b] B-3054, 35695,[b] B-3704, B-766, 96.0509, 97.0147,[b] 97.0152, 97.0190, 99.0512, 99.1232, O157:H43	35695, 96.0509, 97.0152, 97.0190	B-14573, B-2783, B-3054, 35695, B-3704, B-766, 96.0509, 97.0147,[b] 97.0152, 97.0190, 99.0512, 99.1232, O157:43	B-14573, B-2783, B-3054, 35695, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.1232
E. coli O157:H7 OK	(72.89 ± 11.62)	(75.21 ± 13.67)	(67.82 ± 13.24)	(73.63 ± 9.97)	(68.22 ± 8.91)
	B-14573, B-2783,[b] B-3054, 35695,[b] B-766, 97.0147, 97.0152, 99.0512, 99.1232[b]	B-14573, B-2783,[b] B-3054, 35695,[b] B-766, 96.0509, 97.0147,[b] 97.0152, 97.0190, 99.0512, 99.1232	B-14573, 25922, B-2783, B-3054, 35695,[b] B-3704, B-766, 96.0509, 970147, 97.0152,[b] 97.0190, 99.0512, 99.1232	B-2783, 35695, 96.0509, 97.0147, 97.0152, 99.0512, 99.1232	B-14573, B-2783, B-3054, 35695, B-766, 96.0509, 97.0147, 97.0152, 97.0190, 99.0512, 99.132
Salmonella Montevideo G4639	(35.14 ± 6.48)	(46.50 ± 6.64)	(38.19 ± 3.77)	(47.11 ± 73)	(40.83 ± 3.50)
	11775, B-14573,[b] 25253, 25922, B-2783,[b] B-3054, 35695,[b] B-3704, B-766,[b] 96.0509, 97.0147,[b] 97.0152,[b] 97.0190, 99.0512, 99.1232,[b] O157:H43	11775, B-14573,[b] 25253, 25922, B-2783[b] B-3054, 35695,[b] B-3704, B-766, 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190, 99.0512, 99.1232,[b] O157:H43	11775, B-14573, 25253, 25922, B-2783, B-3054, 35695,[b] B-3704,[b] B-766,[b] 96.0509,[b] 97.0147, 97.0152, 97.0190,[b] 99.0512, 99.1232, O157:H43	B-14573, 25922, B-2783,[b] B-3054, 35695,[b] B-3704, B-766, 96.0509, 97.0147,[b] 97.0152,[b] 97.0190, 99.0512,[b] 99.1232,[b] O157:H43	11775, B-14573, 25253, 25922, B-2783[b] B-3054, 35695, B-3704, B-766,[b] 96.0509, 97.0147,[b] 97.0152,[b] 97.0190, 99.0512,[b] 99.1232, O157:H43
Salmonella Poona RM 2350	(23.37 ± 2.12)	(24.51 ± 3.24)	(26.70 ± 2.91)	(23.54 ± 6.12)	(24.01 ± 1.99)
	11775, B-14573,[b] 25253, 25922,[b] B-2783,[b] B-3054,[b] 35695,[b] B-3704,[b] B-766,[b] 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43[b]	11775, B-14573,[b] 25253, 25922,[b] B-2783,[b] B-3054,[b] 35695,[b] B-3704,[b] B-766,[b] 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43[b]	11775, B-14573,[b] 25253, 25922,[b] B-2783[b] B-3054, 35695,[b] B-3704,[b] B-766,[b] 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43	11775, B-14537,[b] 25253, 25922, B-2783[b] B-3054,[b] 35695,[b] B-3704,[b] B-766, 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43[b]	11775, B-14573,[b] 25253, 25922, B-2783,[b] B-3054,[b] 35695,[b] B-3704, B-766,[b] 96.0509,[b] 97.0147,[b] 97.0152,[b] 97.0190,[b] 99.0512,[b] 99.1232,[b] O157:H43

[a] All *E. coli* strains, identified by strain numbers (see Table 1). Values in parentheses are the mean ± standard deviation $D_{60°C}$ (in seconds) for each pathogen in each growth medium.
[b] Strains that had significantly greater ($P < 0.05$) heat resistance than did the pathogenic strain.
[c] *E. coli* NRRL B-2783 contains the O157 gene, and O157:H43 strains were not considered potential surrogates. These strains are included for comparison purposes only.

ment and survival on apple surfaces, resistance to H_2O_2 wash treatment with and without storage at 4°C, growth on selective media, and thermal resistance, all situations where the strains were exposed to stressful challenges. Several strains had attachment and thermal resistance profiles similar to those seen with *E. coli* O157:H7 and *Salmonella* and would, therefore, be suitable surrogate organisms for these pathogens under such test conditions. The phenomenon of different strains of a particular organism having significantly different responses to the same stress has been reported before. Thermal resistance at 55 or 60°C of 17 different *E. coli* O157:H7 strains grown under same conditions varied significantly *(22)*. Buchanan and Edelson *(6)* found different thermal resistance profiles for three different strains of *E. coli* O157:H7 heated at 58°C following growth in TSB+G and TSB−G. Thermal resistance values of *E. coli* O157:H7 SEA 13B88 in the present study following growth in TSB+G or TSB−G were 3.7- and 2.6-fold lower than those values previously reported by Buchanan and Edelson *(6)* for the same strain grown under similar conditions. These researchers determined thermal resistance at 58°C in BHI whereas we used 60°C in PW in this study. Although thermal resistance at 60°C would be lower than that at 58°C, BHI contains more solids than does PW, which could offer protection to the cells and thus result in an increase in thermal resistance *(2)*. Sapers et al. *(19)* applied sanitizing agents, including H_2O_2, to apples inoculated with three different generic *E. coli* strains and one *Enterobacter* strain, and found significant variations in strain responses. These data further underline the necessity to be careful in the choice of nonpathogenic surrogate organisms.

There was a significant synergistic interaction between storage time and treatment (cell populations on apples decreased following storage at 4°C), reiterating the bactericidal effects of storing potentially contaminated fruit at refrigeration temperatures *(1, 15)*. Also, survivors were more resistant to H_2O_2 wash treatment following storage at 4°C (Table 4), which could be due to biofilm formation *(4)*, physiologic adaptation to stress, or survival of stress-resistant subpopulations of the organism. Although the H_2O_2 treatment significantly reduced bacterial numbers, the mean reduction was only in the range of 1 log CFU. Reduction in this range may have been achievable simply by rinsing the fruit in water, although this treatment was not included in the present study. Sapers et al. *(17, 18)* reported 1- to 2-log reductions with comparable H_2O_2 washing treatments and found greater reductions with H_2O_2 washes applied at higher temperatures (50 to 60°C).

Potential surrogate organisms for *E. coli* O157:H7 and *Salmonella* emerging from these studies include *E. coli* B-14573, ECRC 97.0147, ECRC 97.0152, ECRC 97.0190, and ECRC 99.0512 strains. These strains do not differ significantly from the pathogenic strains in their counts on either recovery or selective media (data not shown), and attachment counts were equivalent or higher than those obtained for the *E. coli* O157:H7 and *Salmonella* strains (Table 4). Further examination of the data reveals other examples of potential surrogates that differ from *E. coli* O157:H7 and *Salmonella* strains in only one or two areas and thus may be useful in certain specific situations.

Data presented here indicate that TSB and BHI are the most appropriate growth media. Overall, strains exhibited the shortest generation time, highest maximum population density, and shortest lag phase duration in TSB and in BHI. The highest thermal resistance overall was recorded for strains grown in TSB, although there are some interstrain differences, e.g., *E. coli* O157:H7 SEA 13B88 had significantly higher thermal resistance following growth in TSB+G than following growth in any other medium. *D*-values at 58°C for three different *E. coli* O157:H7 strains following growth in TSB+G were significantly higher than those following growth in TSB−G *(6)*. Similarly, *D*-values at 60°C for *Pediococcus* sp. cells grown in TSB (containing 0.5% glucose) were significantly higher than those for cells grown in tryptone glucose yeast medium (containing 0.1% glucose) *(2, 3)*. However, thermal resistance following growth in standard formulation TSB was not evaluated in these studies. Although growing the cells in TSB+G as an acid adaptation procedure may cause cell injury *(6, 14)*, in a previous study *(5)* a large proportion of such acid-adapted cells (50 to 90%) were stressed during growth to stationary phase. Such stress was likely insufficient to affect growth in generally supportive environments, as can be seen from the growth data for strains in the present study. However, the production of excess acid during glucose metabolism may have decreased thermal resistance of surviving cells to heating at 60°C, particularly for nonpathogenic strains, which do not typically have the high acid resistance reported for *E. coli* O157:H7 *(6)*. However, Annous et al. *(3)* reported that the decrease in pH of growth medium was merely indicative of glucose metabolism and was not correlated with the change in thermal resistance of *Pediococcus* sp. Similarly, in the current study growing cells in the absence of glucose (TSB−G) did not adversely affect growth rates under conditions where no other stresses were present, but the resultant cells were significantly less heat resistant than were cells grown in TSB (containing 0.25% glucose). The relatively low pH of the TSB−GHCl probably led to acid shock of the cells rather than the acid adaptation effect that cells grown in TSB, TSB+G, and BHI encounter *(3, 16)*. Cells added to TSB−GHCl were probably injured during growth in this medium, as indicated by the significantly retarded growth rates, and thus were probably less resistant to subsequent heat treatment. These results further underline the necessity for the development of a well-balanced (universal) growth medium suitable for determinations of thermal *D*-values and other growth characteristics *(2, 3)*.

Overall, higher cell counts were achieved with recovery media than with selective media as has been reported previously *(14)*, although this effect was not significant for all strains. There were no differences in counts among five of the O55 strains and the *E. coli* O157:H7 strains on either recovery or selective media, underlining the relative resistance of these strains to the stresses applied in this study and the suitability of the O55 strains as surrogates for *E. coli* O157:H7.

E. coli ECRC 97.0152 was more similar to the *E. coli* O157:H7 strains than any other strain tested, although any of these O55 strains probably would be a good surrogate for *E. coli* O157:H7. Clonal relationship studies have established that the O157:H7 serotype is closely related to a group of O55 strains associated with infantile diarrhea *(23)*. The O157:H7 serotype most likely arose from an O55:H7-like ancestor through genetic recombination events that added virulence genes to a nonvirulent *E. coli* genome *(23)*. This scenario may explain the close correlation between the observed characteristics of the O55 strains and those of the O157:H7 strains in the present study.

Thermal resistance profiles observed in this study largely agreed with those reported in the literature *(6, 9, 22)* and were the only area in this study where a large degree of separation was observed between *E. coli* O157:H7 and *Salmonella* strains. Any one of the O55 strains mentioned could be a good surrogate organism to use in studies involving commodities, such as melons, that may be contaminated with both *Salmonella* and *E. coli* O157:H7 and that do not readily demonstrate thermal damage *(1)*. Potential surrogate strains with equivalent or higher thermal resistance than pathogenic strains in all growth media tested and thus were considered potential surrogates for these pathogens based on this criterion include *E. coli* ECRC 97.0152, ECRC 97.0147, and ATCC 35695. Other potential surrogate strains that did not differ significantly from pathogenic strains and may be useful in certain specific situations are listed in Table 5. However, although there is some merit in erring on the side of caution by using a surrogate strain with higher thermal resistance than the target pathogenic strain (in this case, *Salmonella*), the use of such a strain could ultimately lead to unnecessary waste of heat energy and overprocessing of the target food product, with associated sensory damage, particularly if there is low likelihood of the presence of an organism as heat resistant as *E. coli* O157:H7. Therefore, difficulty arises when trying to recommend a good surrogate for *Salmonella* from the strains tested. Although *Salmonella* strains tested were among the lowest in terms of thermal resistance in this study, they had relatively high rates of attachment and survival, and very few of the nonpathogenic strains tested had the same resistance patterns. *E. coli* NRRL B-766, for example, was similar in terms of both attachment and heat resistance to *Salmonella* grown in TSB and TSB−G, but employment of this strain would significantly overestimate the heat resistance of *Salmonella* in the other growth media tested. Sensitivity to heat should be of great importance for selecting a surrogate strain in view of the limited efficacy of sanitizing washes and the success of surface pasteurization with hot water *(1, 4, 17)* or steam *(13, 20)*. Although, surface pasteurization resulted in significant improvement in microbiological qualities of some fruits and vegetables and improved the shelf life of cantaloupes *(1)*, it caused thermal injuries to apples *(17)*. Potential surrogates that have heat resistance profiles equivalent to those of *Salmonella* Poona and *Salmonella* Montevidio strains include *E. coli* ATCC 11775, ATCC 25253, ATCC 25922, NRRL B-3054, and NRRL B-766. In this study, we found many other potential surrogates that did not differ significantly from these strains in individual growth media. Annous et al. *(1)* reported similar responses by *Salmonella* Poona RM 2350 or *E. coli* ATCC 25922 on cantaloupes to commercial-scale surface pasteurization. Thus, based on thermal characteristics *E. coli* ATCC 25922 should be an appropriate surrogate for use in evaluating the efficacy of surface pasteurization for reducing and/or eliminating *Salmonella* Poona RM 2350 on cantaloupes in a pilot plant environment. Research with additional strains of *E. coli* O157:H7 and *Salmonella* and possibly other nonpathogenic surrogates is recommended to identify a effective all-purpose surrogate organism for *Salmonella* and *E. coli* O157:H7.

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